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WHAT IS "HETEROSIS"?[1]

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Received April 21, 1948

IT IS not my purpose in this paper to present or to discuss the actual basic causes or the fundamental mechanisms which bring about the increased size, the excessive kinetic energy, the increased productiveness, resistance to disease or to unfavorable conditions of the environment, the "stimulating effects of hybridity," the so-called "hybrid vigor" of English writers and "Luxurieren" of many German writers, which may be observed in this or that cross-bred organism when compared with corresponding inbred or relatively more pure-bred organisms.

My object is merely to consider the spirit and the coverage of the word "heterosis" which I proposed in 1914 to replace the more cumbersome word "heterozygosis" which had been found useful by a few geneticists of that time, myself among them.

What I have to say about the meaning of the word "heterosis" will be better understood if it be kept in mind that I was, as far as I know, the first English-language geneticist to adopt and promote the use of the brilliantly conceived terminological proposals of the Danish plant physiologist, DOCTOR W. JOHANNSEN, which have contributed so much to the precision, clarity and stability of modern genetical terminology. JOHANNSEN'S proposals of "phenotype," "biotype," "gene," "genotype," etc., were launched in 1909 in his "Elemente der exakten Erblichkeitslehre," and in that same year his word "biotype" appeared in the title of one of my Carnegie Institution publications, "*Bursa bursa-pastoris* and *Bursa Heegeri;* biotypes and hybrids."

In proposing the German word "Gen," for whose English form "gene" I was more or less responsible, JOHANNSEN pointed out that DARWIN'S word pangene which had come into use as a specific improvement over the older, ambiguous term "Anlage," was a double expression, consisting of the two parts, "pan" and "gene" of which the first syllable, "pan," was wholly immaterial and irrelevant, and only the second syllable represented the concept which it was desired to express. The word "pangene" had the further disadvantage that it was closely identified with DARWIN'S "provisional hypothesis" with its migrating gemmules and their differential assortment during ontogenetic development. By omitting the non-essential half of the word "pangene," JOHANNSEN arrived at his neat word "Gen" which was thus freed from the Darwinian proposals, and he added the specific stipulation that "das Wort 'Gen' ist völlig

[1] Address of the retiring president of the Torrey Botanical Club, delivered at Hunter College, New York City, January 8, 1948.

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frei von jeder Hypothese"—"the word 'gene' is completely free from every hypothesis!"

It was this completely non-committal quality of JOHANNSEN's proposed terms that impressed me, since they harmonized with the complete open-mindedness that I have always considered essential in scientific research.

The proposal to use "heterosis" instead of "heterozygosis" can be explained in a manner closely paralleling the statements of JOHANNSEN in launching the word "gene," as quoted above, for the word heterozygosis like the word pangene is a double construction of which the first half represents the important significant concept. The words "homozygous" and "heterozygous" were already in general use in reference to Mendelian paired genes, so that the "zygosis" part of the word "heterozygosis" seemed to connote that the effective differences which resulted in the increased size, speed of development, etc., in cross-bred organisms were solely due to heterozygous Mendelian genes, a view that I desired to avoid, even though I proceeded to discuss the problem on the basis of the known distribution of Mendelian genes. The word "heterosis" was chosen in the same spirit as JOHANNSEN's word "gene," namely that it should be *free from every hypothesis*. It represented a group of observable phenomena for which any subsequent student was free to propose his own explanation without thereby being obliged to abandon the word "heterosis."

DOCTOR EAST and I used the word "stimulation" and many others used the expression "hybrid vigor" in discussing these phenomena, but both "stimulation" and "vigor" are ambiguous terms and refer solely to the fact that there is *increased* size or other valuable qualities in the cross-bred as compared with the pure biotypes or the different species or different varieties whose union produced the cross-bred in question. The existence of "stimulation" or "increased vigor" is discovered only when it is translated into visible observable phenomena. Any attempt to distinguish between the visible and the invisible phases of the effects of differences in uniting gametes does not appear to be practically justifiable, and it was intended that the word heterosis should include the entire process from start to finish.

In proof of this intended coverage I will quote from my invitation lecture delivered at Göttingen, Germany, in mid-July, 1914, in which the word "heterosis" was first proposed, and from a paper written at the same period, on "Duplicate genes for capsule form in *Bursa bursa-pastoris*," through which latter medium the word "heterosis" came to the attention of other geneticists; the Göttingen lecture was delivered less than three weeks before the outbreak of World War I, and its publication was delayed for eight years, finally appearing in print in 1922.

I quote verbatim, but in considerably contracted form, from my paper on duplicate genes (1914, pp. 126, 127): "My investigations on the effect of cross and self-fertilization in maize had led me as early as 1907 to the conclusion that . . . hybridity itself,—the union of unlike elements, the state of being heterozygous—has . . . a stimulating effect upon the physiological activities of the organism, which effect disappears as rapidly as continuous breeding

reduces the progenies to homozygous types. . . . There is some danger of misconception due to the fact that all discussion of the stimulus of hybridity have taken as their starting point, for the sake of simplicity, the typical Mendelian distribution of the germinal substances. The essential features of the hypothesis may be stated in more general terms, as follows: The physiological vigor of an organism as manifested in its rapidity of growth, its height and general robustness, is positively correlated with the degree of dissimilarity in the gametes by whose union the organism was formed. . . . The more numerous the differences between the uniting gametes—at least within certain limits —the greater on the whole is the amount of stimulation. . . . These differences need not be Mendelian in their inheritance. . . . To avoid the implication that all the genotypic differences which stimulate cell-division, growth and other physiological activities of an organism are Mendelian in their inheritance and also to gain brevity of expression I suggest that . . . the word 'heterosis' be adopted."

From the Göttingen lecture I make only the following brief quotation: "Bei der Frage die uns heute beschäftigt, halte ich es für unbedingt notwendig, scharf zwischen den Tatsachen und der daraus abgeleiteten Theorie zu unterscheiden. Mit den empirischen Tatsachen wird man immer zu rechnen haben, während sich die Theorie vielleicht als unzulänglich erweisen wird. Es ist sehr möglich, dass noch andere Gründe für die Heterosis und Inzuchtserscheinungen gefunden werden." Translated this says that "I hold it to be absolutely necessary to distinguish sharply between the facts and the theory derived from them. We must always reckon with the empirical facts, while the theory may prove itself to be inadequate. It is very possible that still other bases may be found for the heterosis and inbreeding phenomena."

Other relevant statements could be quoted from this lecture, but since their meaning is in close agreement with the statements above quoted from the paper on duplicate genes it does not appear necessary to repeat them here. These quotations leave no doubt that I offered the word "heterosis" to cover the real, observable phenomena and that it was definitely intended to include cases in which the effective differences between uniting gametes might be due to anything else than Mendelian genes, as well as the differences caused by such Mendelian genes, if the latter were not individually analyzable as Mendelian genes. I have not the least doubt that if I had known at that time the striking phenomena which our brilliant colleague, DOCTOR B. O. DODGE, has discovered in Neurospora, and has discussed under the expression "heterocaryotic vigor," I would have enthusiastically presented them as an example of heterosis which was associated with a mechanism clearly different from that which produced the typical Mendelian distribution of paired genes. In Neurospora the heterosis is evidently the result of the coexistence and interactions of two unlike whole nuclei operating in a common cytoplasmic mass. I can see no reason to assume that the fundamental nature of the phenomena here manifested is different from the increased activity which results when two unlike nuclei fuse and rearrange their unlike elements in the organization of a single

nucleus. In either case the result is assumed to be due to the fact that elements of unlike constitution are brought into sufficiently close association that effective interactions can and do take place.

That my Göttingen lecture was from the time of its publication understood by competent biologists exactly as here stated, can be substantiated by reference to the abstracts published in "Botanical Abstracts" and in "Botanisches Centralblatt," immediately after the printing of this lecture. In "Botanical Abstracts" DR. D. F. JONES says: "The increase in size and vigor resulting from crossing is called heterosis." and in "Botanisches Centralblatt," DR. ELISABETH SCHIEMANN states: "Der Verf. nimmt an, dass bei heterozygoter Konstitution das Protoplasma grössere Fähigkeit besitzt, und bezeichnet die durch diese erhöhte physiologische Fähigkeit bedingte grössere Wüchsigkeit und Ertragsfähigkeit als Heterosis."

A review of other relevant literature since that time shows a general agreement with the author as to what is to be understood by the word "heterosis," and the word is correctly defined in the MERRIAM-WEBSTER *New International Dictionary*, Second Edition. It defines "heterosis" as "The greater vigor or capacity for growth frequently displayed by crossbred animals or plants as compared with those resulting from inbreeding." This same great standard dictionary defines "Hybrid vigor" as "Vigor resulting from hybridity. specif. heterosis."

This synonymy between "heterosis" and "hybrid vigor" is obvious in those cases in which the expression "hybrid vigor" can be appropriately used, and this was clearly recognized and strongly supported by EAST and JONES, the two geneticists of that time who can be recognized as best equipped to speak authoritatively and critically on the meaning of these terms. In their admirable book (1919) on "Inbreeding and outbreeding: their genetic and sociological significance," Chapter VII carries the caption "Hybrid vigor or heterosis," and the context shows decisively that the "or" of the heading signified the synonymy of these terms, not an antithesis between them. In the writings of most other authors the same synonymy has been in evidence, and only rarely has an expression slipped in, which implied that an author considered heterosis to be something more fundamental than hybrid vigor. It seems unfortunate in this connection that W. GORDON WHALEY (1944) should state in "Botanical Review" in his otherwise admirable compilation and discussion of heterosis, that "rather erroneously this term 'heterosis' has become established in the literature as a synonym for hybrid vigor. By the original definition 'heterosis' refers to the developmental stimulation resulting by whatever mechanism, from the union of different gametes. 'Hybrid vigor' denotes the manifest effects of heterosis." I hope I have in the foregoing quotations completely refuted this interpretation of what was involved in "the original definition."

It may be noted that I never used the words "hybrid vigor" in the presentation of my researches in which heterosis was involved. My preference for "heterosis" to its synonym "hybrid vigor" may be explained by the fact that my unexpected discovery and demonstration of this phenomenon were made within the genetical materials included in a small population of white dent

maize which was initially assumed to be genotypically uniform. All of my inbred lines were started from plants in this original population whose sole recognized differences consisted in the possession of different numbers of rows of grains on the female spikes or ears. Although I referred to the crossing in this material as hybridization in accord with general modern genetical usage, there was great contrast between these crosses of biotypes derived from a uniform strain of the single species *Zea mays* L. and the older species hybrids in which hybrid vigor had been frequently so strikingly presented. For this reason it seemed more appropriate in this case to speak of heterosis than of hybrid vigor. This may indicate that "heterosis" has a slightly more extensive coverage than "hybrid vigor," that is, that while all hybrid vigor is heterosis, not all heterosis can be with equal propriety termed "hybrid vigor." This is particularly the case in Neurospora and other groups of Fungi in which the juxtaposition of unlike elements is brought about by nuclear migrations, not by cross-fertilization.

Limitations of time do not permit me to essay an adequate consideration of the effective causes of heterosis, but I am impelled by the opportunity this occasion offers, to make several statements on this phase of the subject. I hold that heterosis is not a unitary phenomenon, but a complex series of phenomena for which no single cause or mechanism can be properly assumed to apply to all cases. The early hypothesis of DOCTOR EAST and myself that heterogeneity of the protoplasmic materials results in greater physiological effectiveness need not be discarded because of any seeming plausibility of DOCTOR JONES'S hypothesis (1917) that many linked size factors are dominant in character, and that increased vigor may be produced by the increased number of such dominant favorable factors which will be present when paired chromosomes are of unlike constitution, as they are likely to be when they have come from unlike parents, than when they are derived from like parents.

In relation to JONES'S hypothesis, it is important to keep in mind that the phenomenon of dominance in size factors is a demonstrable reality only when there is a single gene whose presence results in a relatively considerable change in size, as in MENDEL'S cross between tall and dwarf peas and in many other examples of hereditary dwarfism found by other investigators in both plants and animals.

When NILSSON-EHLE (1909), followed closely by EAST (1910), discovered the first examples of duplicate genes, and therefrom developed a Mendelian interpretation of quantitative-factor inheritance, it was assumed by EAST and accepted by most other geneticists as a necessary condition for such interpretation, that we pre-suppose the absence of dominance in the genes producing the quantitative characters. In an important paper that has been long since overlooked and apparently completely forgotten, I showed, more than 27 years ago (SHULL 1921), that we need not deny the occurrence of dominance of size factors in the Mendelian interpretation of quantitative inheritance. The small effects produced by any individual size modifying "quantitative" gene compared with the fluctuations produced by environmental and developmental variations, make the question whether any such gene produces as great

effect in the heterozygous phase as in either homozygous phase wholly academic, since it can never be settled; the correct scientific attitude in such a situation is one of skepticism, when any one asserts either that such quantitative paired genes are *generally* dominant and recessive, respectively, or that they *generally* have an intermediate effect in the heterozygous phase as compared with the two alternative homozygous phases.

In view of the complete absence of the conditions necessary for a determination of the occurrence of dominance versus intermediacy of size factors in their heterozygous phase it is regrettable that one can read in certain recent papers of RICHEY (1945) and others that the cumulative effects of dominant favorable genes is "the generally accepted" explanation of hybrid vigor. Such a statement seems to imply that the heterosis problem has been completely solved and requires no further investigation. To point the fallacy of such a conclusion I call attention to an important paper by EAST, published in 1936 only two years before his untimely death. DOCTOR EAST was the greatest authority on the subject of heterosis, and his paper under the simple title "Heterosis" is the best and most comprehensive treatment of the subject now available. In this paper EAST considered and specifically rejected the hypothesis that dominant favorable factors play any role in heterosis. He offered a new suggestion based, like JONES's hypothesis, on the linkage of quantitative genes, but proposing the hypothesis that heterosis is due to partially additive effects of multiple alleles. JONES himself (1945) has recently demonstrated that apparently degenerative changes in six mutations in homozygous inbred lines of maize have produced notable degrees of heterosis when these new mutant forms are crossed back to the inbred parent strains. These results seem to be in essential agreement with the later hypothesis of EAST, above mentioned. They do not support JONES's reiteration that "heterosis is interpreted as an accumulative effect of favorable heredity from both parents." Instead of the heterosis being due in these cases to the accumulation of favorable dominants, they are clearly due to the occurrence of *unfavorable recessives*. CASTLE (1946) offers a new and somewhat different explanation of the heterosis which JONES has demonstrated in his six new "degenerate" mutants from inbred strains. CASTLE points an analogy to the "killer gene" found by SONNEBORN in Paramecium, and suggests that the heterosis is due to the (anaphylaxis-like) sensitization of the new recessive "degenerate" genes by the parental dominant gene at the same locus.

The several hypotheses which have been proposed for the explanation of heterosis are in the main not mutually exclusive: (a) the entrance of a sperm into a "foreign" cytoplasmic environment may in some cases produce an initial favorable reaction which may manifest itself in the F_1 and not be repeated or not repeated in the same degree in F_2 and subsequent generations (A. F. SHULL 1912); (b) protoplasmic heterogeneity may favor increased metabolic activity (E. M. EAST and G. H. SHULL); (c) linked dominant favorable factors may confer some advantages in heterozygotes as compared with homozygotes (D. F. JONES, F. D. RICHEY, and others); (d) Multiple alleles produced by repeated mutations at single loci may produce additive effects when unlike

alleles are present (E. M. EAST); (e) JONES's (1946) demonstration that striking heterosis resulted from back-crossing of several mutant strains to the parental homozygous inbred strain, gives still another mechanism for the production of heterosis, for here it is not the accumulation of favorable dominants, but the rare occurrence of an unfavorable recessive that induces heterosis; and (f) for this case CASTLE (1946) proposes a sensitization of the non-mutated parent gene by its mutated allele, or *vice versa*, of the nature of an anaphylaxis, that produces the heterotic effect. Since these mechanisms are not mutually exclusive two or more of them may be jointly involved in any single example of heterosis.

Any attempt to restrict "heterosis" to any one of these possible causes of increased vigor in cross-bred organisms, would render the term heterosis relatively useless and that would be a distinct misfortune for the cause of stability in genetic terminology. As an example of such restriction I quote from DR. PAULA HERTWIG (1936, p. 49, 50): "Dass gerade bei Artbastarden von Säugetieren besonders kräftige F_1-Tiere häufig sind, lässt darauf schliessen, dass wir es hier kaum mit Heterosis im eigentlichen Sinne zu tun haben. Das stärkere Wachstum wird wohl weniger darauf zurückzuführen sein, dass eine grössere Heterozygotie und Anhäufung von dominanten Faktoren erreicht worden ist, es scheint mir vielmehr wahrscheinlicher, dass es sich um die Wirkung einiger weniger komplementärer Faktoren handelt, die auf das inkretorische System einwirken und dadurch wachstumsfördernd sind." If DOCTOR HERTWIG is correct that complementary genes are producing a favorable growth-enhancing modification of the secretory system, I interpret that as merely recognizing such complementary factors as a part of the heterotic mechanism, not as justification for the suggestion that no heterosis is involved.

A similar statement can be made regarding the recent observation by ROBBINS and students, that specific growth promoting substances occur in the excised roots of heterozygotes that are not present in either of the homozygous parents.

The fundamental consideration for the objectives of the present paper is that, although five or six sources of heterosis have been proposed and discussed by different authors, and others may be offered in the future which have not yet been dreamed of, the non-committal quality of the word allows authors to continue to use the word "heterosis". If the spirit in which the word was proposed is maintained, as urged in this paper, heterosis will always continue to be a useful genetical term, and new researches will be encouraged to determine the factors involved in each specific example.

POSTSCRIPT

Since this address was written and delivered, my attention has been drawn to a misuse of the word heterosis, which I consider unfortunate and unnecessary, and which I hope will be discontinued. This is the use of the expression "negative heterosis" for a situation which has no apparent or obvious relationship with the phenomena for which the word "heterosis" was proposed and has been generally adopted. I can find no more justification for the expression

"negative heterosis," than there would be for calling hydrophily "negative xerophily," or photosynthesis "negative respiration."

By definition, heterosis is the *increase* of size, yield, vigor, etc. If there is no such *increase*, there is no heterosis. A decrease in size, vigor, etc., should be treated as another phenomenon, since it is not clear that any of the causes involved are the same as those which produce an increase of these functions.

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DELAYED PHENOTYPIC EXPRESSION OF SPONTANEOUS MUTATIONS IN ESCHERICHIA COLI*

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INTRODUCTION

THE quantitative study of mutations occurring at low rates requires an organism that can be grown conveniently in large numbers, and mutations that can be readily detected. Furthermore, where information is desired concerning the effect of a gene change shortly after its occurrence, it is necessary to be able to examine the phenotype of the organism immediately following the change and at intervals thereafter.

Bacteria are superior to higher organisms for these purposes, since (1) large populations can be handled, (2) there are numerous mutants that can be readily detected and counted, and (3) individual organisms result from each cell division, enabling the phenotype to be determined at any time after a gene change. It was the purpose of this investigation to discover the rate of spontaneous mutation of the bacterium *Escherichia coli*, strain *B/r*, from sensitivity to resistance to the phage *T1*, and also, by indirect means, to determine the interval between time of occurrence of the mutation and phenotypic expression.

The reasons for investigating spontaneous mutation rate and time of phenotypic expression are as follows:

Two previously developed methods of estimating mutation rate in bacteria have yielded discrepant results (LURIA and DELBRÜCK 1943). This discrepancy has been ascribed to an error in the assumptions on which one of the methods is based; but, which assumption and which method is in error is not known.

One of the methods rests on the assumption that a gene mutation expresses itself immediately in the individual cell in which it occurs; and one of the possible interpretations of the discrepancy is that this is not the case, but that on the contrary one or more generations of growth are required before the mutation is expressed.

Delayed phenotypic expression, or "cytoplasmic lag" as it has been termed, has been observed in Paramecium (see SONNEBORN 1947); and if something of this nature occurs also in bacteria it is important from the standpoint of understanding gene action.

* The cost of the accompanying tables has been paid by the GALTON and MENDEL MEMORIAL FUND.

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Methods of Previous Workers

The early experiments of LURIA and DELBRÜCK (1943) referred to above will now be considered in detail.

To find out the number of phage-resistant mutants in a phage sensitive liquid culture, the whole culture if its population size is small, or a sample of it if it is large, is spread on agar together with the particular phage under consideration. On incubation, all bacteria that are sensitive to the phage are lysed, leaving only the resistant mutants. Each such mutant will eventually form a separate colony; and from the number of colonies the number of mutants in the liquid culture, or the sample, can be estimated.

By using this technique it is possible to determine the rate of mutation to phage resistance. As stated, two methods have been devised by LURIA and DELBRÜCK (1943). The experimental procedure in both of the methods is to grow a series of similar liquid cultures from small inocula, and to determine the numbers of resistant bacteria in each, as well as in the average population. The two estimates are derived from these primary data, the first using the proportion of cultures in which resistant mutants have appeared, and the second using the average number of resistant mutants per culture.

These methods are of course only strictly applicable where it is possible to eliminate the original type without affecting the mutant type, and where there are no selective differentials between mutant and original types.

In their experiments these test cultures were started with small inocula (50 to 500 bacteria from a growing culture of *E. coli*, strain *B*) and were grown to saturation either in broth or in synthetic medium. (The volume of the cultures was 10 cc in some experiments and 0.2 cc in others, the final numbers of bacteria being of the order of 3×10^{10} and 3×10^{8} respectively.) At the end of growth, samples of the cultures—or in some cases whole cultures—were tested to determine the numbers of bacteria resistant to the phage *T1*.

The cultures from which the inocula were taken contained between 1 and 1,000 resistant individuals per 10^{8} total bacteria. Thus, the chance of introducing a resistant bacterium into the test cultures via the inoculum was small and in the rare event of one being introduced the fact would be indicated by an excessive proportion of resistant bacteria in the fully grown test culture. In practice, any resistant bacteria found at the time of testing—that is, after the cultures are fully grown—will therefore be the mutant offspring of one of the sensitive bacteria in the inoculum.

An estimate of mutation rate per bacterium per division cycle can be obtained if the numbers of mutations, and the average number of cell divisions, occurring in a series of cultures are known. The latter may be calculated from the final population in the series and the former from the proportion of cultures containing no mutants. The greatest accuracy is obtained when this proportion is neither too large nor too small, and the method cannot be used if every culture contains a mutant. Since the proportion of cultures having no mutants is a function of the number of cell devisions in a culture, it may be adjusted by altering the volume of medium, 0.2 cc being the amount used

in the experiments under consideration. To determine this proportion it is of course necessary to test whole cultures, as distinct from samples.

This method will be known as *method 1* throughout the present paper. It should be noted that it is based upon an estimate of the number of resistant clones developing in the series (this estimate being obtained from the proportion of cultures in which no resistant mutants have developed), and that it takes no account of the numbers of individuals in these clones at the end of the growth. For the purpose of this paper, the term "mutant clone" will refer to those individuals carrying genetic factors for phage resistance which have a common origin in a single mutation. Within a mutant clone individuals which are phenotypically resistant to phage will be collectively termed a "resistant clone." A culture may contain one or more mutant clones of varying age.

The second method of LURIA and DELBRÜCK uses the average number of resistant bacteria in a series of similar cultures and calculates mutation rate from this value, the average population, and the number of cultures. The number of mutants arising during the growth of a culture is of course, on the average, a function of the mutation rate. But there are very large variations in the number of mutants present in different cultures grown under identical conditions, these being due to chance variations in the time of occurrence of the mutations. Thus the occasional occurrence of a mutation early in the growth of a culture, at a time when the population is small, will result in a much higher than average number of mutants in that culture. Because of these statistical fluctuations, mutation rate cannot be calculated from the number of mutants in a single culture started from a small inoculum. It can, however, be estimated from the average number of mutants in a series of similar cultures; and the mathematical details of the method have been worked out by LURIA and DELBRÜCK (1943). This method will be known as *method 2* throughout the present paper. It differs from method 1, which utilizes the number of resistant clones occurring in liquid cultures, in that it takes into consideration the number of resistant individuals. Furthermore, in the event of any change in the mutation rate during growth, method 2 would give an average of the mutation rates obtaining in each of the generations during which mutations had occurred—equal weight being given to the early generations when the population and the number of mutations occurring were small, and to the later generations when both these values had increased. In contrast to this, in method 1 changes in the mutation rate during growth would give an estimate strongly biased in favor of the rate obtaining during the later period when the population and the absolute number of mutations occurring was large. Moreover, a delay in the phenotypic appearance of a mutation would reduce the rate as estimated by method 1, because recent mutations would not be detectable. The rate as estimated by method 2 would be affected less, since early mutations, which have a greater number of generations in which to become phenotypically resistant, are represented by larger numbers of descendants than are the later mutations. The possibility that mutation rate is

not constant throughout growth, and the possibility that phenotypic resistance does not appear for one or more generations after mutation to resistance has taken place, will now be considered.

A striking and unexpected finding of the LURIA and DELBRÜCK experiments was that the rates estimated by these two methods differed by a considerable factor, that from method 1 (utilizing the number of resistant clones developing in liquid cultures) being lower than that from method 2 (utilizing the average number of resistant individuals per culture). The averages of their estimates are $.32\times10^{-8}$ and 2.4×10^{-8} per bacterium per division cycle, respectively. This difference has since been confirmed by DEMEREC and FANO (1945), who have in addition shown that it is not peculiar to experiments using *T1* but is also true of rates of mutation to resistance to other phages (*T3*, *T4*, *T5*, *T6*, and *T7*) when estimated by methods 1 and 2.

A statistical bias in method 2 (which gives the high estimate) may contribute to the discrepancy, but the work of LURIA and DELBRÜCK suggests that its contribution is small. (For a discussion of this point the reader is referred to the original publication. Also, a method of estimating mutation rate, which avoids this source of error will be considered in a later section of the present paper.)

Since the two methods will give the same estimate if, and only if, (1) the rate of mutation is constant throughout growth and (2) the occurrence of a mutation gives rise to a phenotypic mutant without delay, it was concluded that one of these two conditions did not obtain. There was no critical evidence to indicate which one, however, since the discrepant estimates could be explained by assuming either a high mutation rate during the greater part of the growth period, dropping during the last few divisions, or a delay of one or more generations between mutation and phenotypic expression.

LURIA and DELBRÜCK did not favor the latter interpretation, since a fixed delay of one or more generations before the development of phenotypic resistance would mean that mutant clones would number two or more individuals at the time when phenotypic resistance appeared. It was therefore assumed that cultures with just one resistant individual would be rare if there were a delay; and these, instead of being rare, had been observed in considerable numbers (see LURIA 1946). It has been pointed out, on the other hand, that if some lines of descent within the clone were to develop resistance earlier than others, mutation plus delay could give rise to cultures having only one phenotypically resistant individual (SONNEBORN 1946). Thus the assumption of a delay is permissible provided it is also assumed that phenotypic expression is earlier in some lines of descent than in others within the same mutant clone.

Two alternative possibilities therefore exist: (1) that of a relatively high mutation rate during all but the last few generations, and (2) that of a variable delay in phenotypic expression. The possible significance of the second of these two alternatives should be considered. If there is a delay in phenotypic expression, then some cultures which showed no phenotypically resistant bacteria would contain mutants that could not be detected. Also, the end number of resistant bacteria in a culture would represent only part of the genetic mutants

present, some not having developed resistance by the time growth stopped. Thus the mutation rates calculated by methods 1 and 2 would both be underestimated, and the extent of the underestimate would depend upon the magnitude of the delay.

The information available so far sets no limit on the suspected delay or its variability within clones of mutants, and it is even possible that an extreme situation exists in which both are considerable. If this is true, mutation rate is greatly underestimated by the methods outlined.

It should be noted at this point that a delay similar to that suspected in the case of spontaneous mutations does in fact occur in irradiated material (DEMEREC 1946; and DEMEREC and LATARJET 1946). Although there is at present no certainty that spontaneous and induced mutations behave in precisely the same manner, it is of interest to consider the nature of the delay in the one case in which it has been established, that is, in induced mutations.

To determine the time of appearance of induced mutations, DEMEREC irradiated bacteria in liquid suspension, using ultraviolet radiation in some experiments and X-rays in others. These treated bacteria were spread on agar, incubated for varying periods of time to permit cell reproduction, sprayed with phage *T1*, and incubated again until colonies appeared. The spraying caused all susceptible bacteria to be infected and lysed; but where a mutation to resistance had occurred and had been expressed phenotypically, the resulting bacteria continued to grow after phaging, giving rise to one visible colony for each such mutation.

Irradiation caused an enormous increase in the number of resistant clones that appeared during growth, over the number that appeared during the same number of generations in untreated bacteria. The delay between irradiation and phenotypic change was such that less than one percent of the induced mutants appeared prior to the first division, 50 percent appeared after about five divisions, and some did not appear until after 11 or 12 divisions.

These observations give some support to the possibility that there is also a delay in the expression of spontaneous mutations.

The experiments described here were designed to distinguish between this and the alternative possibility of a change in the rate of mutation during growth. They show that there is in fact a delay, and that it is the cause of the discrepant estimates of rate obtained by using the LURIA and DEBRÜCK methods 1 and 2. Some indication of the extent and variability of the delay has been sought, and an attempt has been made to obtain a more accurate estimate of mutation rate.

MATERIALS

The bacterium *Escherichia coli* strain *B/r* was used in these investigations. This is a mutant derived from strain *B* (WITKIN 1946, 1947), and is more resistant than *B* to the action of radiations.

The mutations studied are those resulting in resistance to phage *T1*. There are at least two different categories of mutant: those resistant to *T1* but not to any other of the known phages, and those resistant to phages *T1* and

T5. These mutant categories are designated *B/r/1* and *B/r/1,5* respectively. Within each there occurs a number of morphologically distinguishable colony forms, and it is possible that these represent a number of mutations of dissimilar origin; but in this study no attempt has been made to distinguish between the various types of mutation that give rise to resistance to *T1*.

DISCREPANT ESTIMATES OF MUTATION RATE FROM NUMBERS OF RESISTANT CLONES (METHOD 1) AND OF RESISTANT INDIVIDUALS (METHOD 2)

In view of the possibility that *B/r* may differ from *B* in the rate with which it mutates, rates for *B/r* were determined by each of the LURIA and DELBRÜCK methods, using phage *T1*.

Eight separate experiments were carried out, and for each experiment 25 broth cultures of 0.2 cc were grown. Small inocula were used, and the cultures were incubated for 18 hours, by which time growth had stopped.

The inocula contained approximately ten bacteria per culture in four of the experiments, and approximately 10^4 in the other four. These numbers were small enough so that the chance carry-over of a mutant in the inoculum would be readily detected.

Method 1 was used to calculate mutation rate, a, from the proportion of cultures having no resistant bacteria, P_0, and the average number of bacteria at the end of growth, N, using the formula:

$$a = -(\ln 2)(\ln P_0)/N. \qquad (1)$$

The above formula is derived from formulas (4) and (5) of LURIA and DELBRÜCK (1943), ln being the natural logarithm.

Method 2 was used to calculate mutation rate, a, from the average number of resistant bacteria per culture, r, the average number of bacteria at the end of growth, N, and the number of cultures, C, using the formula:

$$r = (aN/\ln 2) \ln (CaN/\ln 2). \qquad (2)$$

This is derived from formula (8) of LURIA and DELBRÜCK.

The natural logarithm of 2 appears in these formulas because the mutation rate refers to the rate per bacterium per division cycle, as distinct from the rate per bacterial division. The significance of this distinction is best visualized by using a concrete example. If a population of 10^8 bacteria passes through one division cycle and one mutation takes place, the number of bacterial divisions is 10^8 and the mutation rate per bacterial division is 1×10^{-8}. The mean population throughout the cycle, however, is $10^8/\ln 2$, so that the rate per bacterium per division cycle is $\ln 2\times10^{-8}$, which is $.693\times10^{-8}$.

The first of these two methods of expressing mutation rate would be applicable if mutation took place only at the time of cell division, and affected just one of the offspring. The second would be applicable if mutability were continuous throughout the division cycle. In the absence of information on this point the choice is arbitrary, and since the latter method has been used by previous authors its use is continued in this paper to facilitate comparisons.

These considerations are of course based on the assumption that each bacterium divides, an assumption which will be discussed later in the paper.

Methods 1 and 2 have been used with strain *B/r* in order to determine (a) whether, as with strain *B*, estimates obtained by method 1 are lower than those obtained by method 2, and (b) whether the estimates from these two methods are the same for *B/r* as for *B*.

The data from these experiments and the estimated mutation rates are given in table 1. Those obtained using method 1 average $.40 \times 10^{-8}$ and, those using method 2 average 3.6×10^{-8}.

It will be seen from table 9—in which the results of previous workers, using strain *B*, have been quoted—that the discrepancy between the estimates of mutation rate given by the two methods as applied to *B/r* is similar to the discrepancy using strain *B*. It is also evident that the estimate of mutation rate for strains *B/r* and *B* are similar.

THE ELIMINATION OF A POSSIBLE UPWARD BIAS IN METHOD 2 BY THE USE OF LARGE INOCULA (METHOD 3)

The formula for calculating mutation rate from the average number of mutants per culture (method 2) disregards the early divisions, when the population is small and it is unlikely that a mutation will occur. The divisions which enter into the calculation are those occurring after an arbitrary time, this time being chosen so that on the average one mutation will occur prior to it in the whole series of cultures. LURIA and DELBRÜCK point out that the chance occurrence of this early mutation might account for part of the discrepancy between the estimates of rate obtained with the two methods. For a detailed discussion of this point the reader is referred to their paper.

It was therefore necessary to arrive at an estimate which, like that obtained by method 2, would utilize the number of resistant bacteria arising during growth in liquid culture, but which would not be biased by the chance occurrence of early mutations. This was done by growing the test cultures from inocula of sufficient size to ensure that an appreciable number of mutations would take place during the first division. Since much of the statistical fluctuation in end numbers of resistant bacteria is thus eliminated, mutation rates may be estimated from single cultures. (An experiment similar to this has been proposed by SHAPIRO 1946.)

The method can be used only if the proportion of resistant bacteria in the inoculum is small, since otherwise the relatively small increase due to mutation during growth could not be accurately determined. To serve as inocula, therefore, cultures containing very small proportions of resistant bacteria were chosen.

Five 50 cc and five 300 cc aerated cultures were grown from inocula of 2.6×10^9 and 2.1×10^8 bacteria, respectively. Synthetic medium, the M-9 of ANDERSON (1946), was used because the bacteria can be grown to a higher number per unit volume in it than in broth. In two separate sets of experiments growth resulted in increases in the numbers of individuals of approximately a hundredfold and three thousandfold, respectively.

TABLE 1

Estimates of mutation rate of B/r to resistance to phage T1, using the methods of LURIA *and* DELBRÜCK *(1943) (methods 1 and 2 in the present paper), calculating from the number of cultures with no resistant bacteria, and from the average number of resistant bacteria per culture, respectively, in series of similar cultures started from small inocula.*

EXPERIMENT	A	B	C	D	E	F	G	H
Inoculum (no. of bact.)	10	10	10	10	10^4	10^4	10^4	10^4
Number of cultures	25	25	25	25	25	25	25	25
Vol. of cultures, cc	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Culture Number	Number of Resistant Bacteria							
1	140	2	2	1	1	1	134	4
2	0	41	57	5	9	21	18	1
3	0	45	1	1	2	34	34	0
4	43	11	6	4	0	287	380	6
5	60	8	1	4	242	46	3	149
6	13	1	1	71	2	33	15	176
7	48	30	1	5	12	23	2	31
8	3	1	2	12	0	3	6	1
9	4	1	14	1	2	102	4	0
10	36	0	0	1	2	84	2	48
11	55	11	3	49	11	148	5	0
12	447	0	139	9	29	0	18	45
13	1	1	40	0	17	120	0	3
14	9	7	142	0	42	10	33	2
15	27	1	1	1	7	4	725	134
16	14	4	154	1	15	0	1	25
17	30	6	3	60	14	0	5	34
18	160	15	4	37	45	13	0	14
19	231	9	1	1	110	0	0	131
20	35	0	158	7	32	376	118	22
21	37	1	5	44	0	1	1	60
22	0	21	1	18	32	133	15	0
23	1	0	0	0	8	4	0	158
24	8	27	2	12	36	42	151	6
25	3	221	0	79	0	4	9	263
Method 1								
Cult. with no resist. b.	3	4	3	3	4	4	4	4
Bact. per cult., $\times 10^8$	3.1	4.6	2.5	2.8	4.2	3.7	3.2	3.8
Mutation rate, $\times 10^{-8}$	.42	.28	.59	.53	.31	.34	.40	.33
Method 2								
Av. resist. b. per cult.	56.1	18.5	29.5	16.9	26.8	59.5	68.6	52.6
Mutation rate, $\times 10^{-8}$	5.1	1.5	3.8	2.1	2.1	4.5	5.8	3.9

Assays were made of total bacteria and of numbers of resistant individuals in the inocula and in the fully grown test cultures. These four values are designated N_1, r_1, N_2 and r_2, respectively. For the sake of accuracy, five or ten independent assays were made in each case. The number of generations of growth, g, was determined for each of the cultures from the values N_1 and N_2.

Where the mutation rate per bacterium per division cycle is a, and the rate per bacterial division is a/(ln 2), the proportion of mutant bacteria in a culture will rise during growth by a fixed increment of a/2(ln2) per generation, provided the inoculum is of sufficient size so that there are no appreciable statistical fluctuations in the numbers of mutations occurring in the first division. Thus mutation rate can be obtained from the formula

$$a = 2(\ln 2)(r_2/N_2 - r_1/N_1)/g. \quad (3)$$

TABLE 2

Mutation rates of B/r to resistance to phage T1, calculated from the increase in the proportion of resistant bacteria in cultures grown from large inocula (method 3 of this paper). Short growth period.

TEST CULTURE	A	B	C	D	E	INOCULUM
Replicate assays	10	10	10	10	10	10
Inoculum, bact., $\times 10^8$	26	26	26	26	26	—
Vol. of culture, cc	50	50	50	50	50	—
Incr. in no. of bact.	114×	75×	112×	135×	46×	—
Resist. bact., $\times 10^{-8}$	19.6	20.8	24.4	20.9	15.4	7.0
Standard deviation	3.7	4.7	2.6	3.5	2.7	1.3
Generations growth	6.8	6.2	6.8	7.1	5.5	—
Incr. resist. b., $\times 10^{-8}$	12.6	13.8	17.4	13.9	8.5	—
Mutation rate, $\times 10^{-8}$	2.6	3.1	3.5	2.7	2.1	—

TABLE 3

Mutation rates of B/r to resistance to phage T1, calculated from the increase in the proportion of resistant bacteria in cultures grown from large inocula (method 3 of this paper). Long growth period.

TEST CULTURE	F	G	H	I	J	INOCULUM
Replicate assays	5	5	5	5	5	10
Inoculum, bact., $\times 10^8$	2.1	2.1	2.1	2.1	2.1	—
Vol. of culture, cc	300	300	300	300	300	—
Incr. in no. of bact.	3600×	2910×	2780×	2770×	3470×	—
Resist. bact. $\times 10^{-8}$	37.3	30.3	29 8	42.4	24.7	4.4
Standard deviation	6.9	3.9	3.5	6.2	5.6	1.1
Generations growth	11.8	11.5	11.5	11.5	11.8	—
Incr. resist. b., $\times 10^{-8}$	32.9	25.9	25.4	38.0	20.3	—
Mutation rate, $\times 10^{-8}$	3.9	3.1	3.0	4.6	2.4	—

The data from two sets of experiments (involving 6 generations and 11 generations of growth) are given in tables 2 and 3, together with the mutation rates obtained. These rates average 2.8×10^{-8} and 3.4×10^{-8}, respectively—values which are not appreciably different from each other, or from those obtained by method 2, using the average number of resistant bacteria per culture in cultures started with small inocula.

Since there is no question of a statistical bias in calculating mutation rate by method 3, the agreement may be interpreted as confirming the higher

value obtained by means of method 2. This is important, inasmuch as there has been no estimate of the magnitude of the bias in method 2, or of the extent to which the discrepancy between methods 1 and 2 is a product of it.

The above experiments demonstrate that this discrepancy is due to biological rather than to statistical causes.

There are two reasons for measuring mutation rate over periods of 6 and of 11 generations. First, had it been measured over the shorter period only, it would be possible for the results to be biased by a high mutation rate during the first generation or so. The obtaining of similar estimates of rate from both a short and a long period, however, eliminates this as an appreciable source of error.

Second, formula (3) assumes that the mutants multiply at the same rate as the parent strain. That this assumption is approximately true is indicated by certain experiments of of LURIA and DELBRÜCK and later of DEMEREC and FANO. Somewhat more critical evidence however is obtainable from experiments such as the above in which close agreement between estimates of mutation rate from short and from long periods of growth indicate that there is no appreciable bias arising from differential increase during logarithmic growth. A mathematical demonstration of this will be found in a paper by SHAPIRO (1946).

For present purposes it is sufficient to state the argument in general terms. From formula (3) it is evident that mutation acting alone causes the proportion of mutants r/N to rise arithmetically with each successive generation. If, however, the mutants were to increase more (or less) rapidly than the parent strain there would be superimposed upon this an exponential increase (or decrease) in r/N, and estimates of rate obtained using formula (3) would tend to rise (or fall) correspondingly with increasing periods of exponential growth. Thus it is clear that close agreement between estimates of mutation rate over periods of 6 and 11 generations constitutes evidence that the high estimates obtained using methods 2 and 3 are not the result of a differential favoring the mutant during periods of logarithmic growth.

It would be quite possible to supplement the above evidence on the relative rates of increase of mutant and non-mutant strains, by preparing mixed cultures and determining the change in proportion which takes place as the result of competitive growth, as in the above mentioned experiments of LURIA and DELBRÜCK, and DEMEREC and FANO. However the results of such experiments are not entirely critical in the case of the spontaneously occurring mutants, since the mutant strain must first be selected by growing in the presence of phage, and then must be freed from all phage particles by suspending a resistant colony in liquid, streaking the suspension on agar, and incubating until visible colonies are formed, the process being repeated a number of times. Such prolonged growth offers considerable opportunity for further mutation and selection, and it is impossible to be certain that the strain which is finally obtained will not have changed with regard to its ability to compete with the non-mutant.

This difficulty may be avoided, however, by the use of radiation induced

mutants of a similar kind, since these occur much more frequently, and cultures containing mutants and non-mutants in suitable proportions for competitive growth experiments can be obtained without the necessity for first isolating the mutant strain. Evidence has been obtained in this manner in connection with a separate study (NEWCOMBE and SCOTT) which will be published later. Six independent mutant clones were tested over prolonged periods of growth (18 to 20 generations) in competition with the corresponding non-mutant strain. In some cases the proportion of mutants was found to remain unchanged and in others to have declined slightly, the factors of change ranging from 1.20 ± 40 down to $0.16 \pm .05$. In no case did the proportion of mutants increase significantly during growth.

Thus, provided one assumes that the radiation induced mutants are identical to those occurring spontaneously, evidence from this source is in agreement with the above conclusion that the high estimates of mutation rate from methods 2 and 3 cannot be due to a differential favoring the mutants during the period of rapid growth.

These experiments do not eliminate, however, the possibility that during the approach to saturation, when the environment has altered considerably, there may be conditions favoring the mutant strain. An increase in the proportion of mutants during this period of approximately six times, would be sufficient to produce the observed high estimates of mutation rate arrived at from the numbers of individual resistant bacteria.

In order to test this possibility, an experiment was designed in which the conditions associated with the approach to saturation could act repeatedly on a bacterial population passing through approximately five to six generations, thus accentuating any selection.

Five replicate test cultures, each containing in the first instance one cc of broth, were incubated over a period of three days, during which time the amount of liquid medium was doubled at regular intervals and finally brought up to fifty cc. If the approach to saturation in the test cultures used in connection with methods 2 and 3 is accompanied by a six-fold increase in the proportion of mutants, one would expect from the above treatment an increase of much more than six times, in addition to any mutants which arose from spontaneous mutation. Also, estimates of mutation rate obtained from these cultures using method 3, should be very much greater than the corresponding estimates from normal test cultures.

Such estimates of mutation rate from the above experiment are presented in table 4. It will be seen that these are not appreciably increased by keeping the cultures under conditions approaching saturation throughout the whole of the growth period.

In evaluating these data it will be noted that all assays are of numbers of viable bacteria, and that if there is appreciable death due to the conditions of growth, the number of cell generations will be underestimated. This problem is considered in detail in a later section, where it will be shown that the effect of undetected cell death is to increase the estimate of mutation rate obtained, to a value above that of the true rate. Thus the experiment is weighted against

the argument, and the results can be considered as critical evidence that there are no appreciable differentials favoring the mutants during the latter part of the growth of a culture.

The conclusions from the experiments discussed above are applicable to differentials, both of division and of survival, and eliminate all possibility that the widely different estimates of mutation rate obtained using method 1

TABLE 4

Mutation rates of B/r to resistance to phage T1, calculated from the increase in the proportions of resistant bacteria in cultures grown from large inocula (method 3 of this paper). Entire growth under conditions approaching saturation.

TEST CULTURE	K	L	M	N	O	INOCULUM
Replicate assays	5	5	5	5	5	5
Inoculum, bact., $\times 10^8$	29.1	29.1	29.1	29.1	29.1	—
Initial vol. of cult., cc	1	1	1	1	1	—
Final vol. of cult., cc	50	50	50	50	50	—
Incr. in no. of bact.	33×	24×	48×	60×	46×	—
Resist. bact., $\times 10^{-8}$	15.9	15.5	17.2	18.7	21.6	5.0
Standard deviation	2.0	2.9	1.3	1.2	1.8	0.7
Generations growth	5.1	4.6	5.6	5.9	5.5	—
Incr. resist. b., $\times 10^{-8}$	10.9	10.5	12.2	13.7	16.6	—
Mutation rate, $\times 10^{-8}$	4.3	4.6	4.4	4.6	6.5	—

on the one hand, and methods 2 and 3 on the other, are due to such differentials.

Method 3 thus provides critical confirmation of the estimates obtained by method 2.

VARIATIONS IN MUTATION RATE WHICH WOULD BE REQUIRED TO EXPLAIN THE DISCREPANT ESTIMATES FROM METHODS 1 AND 2

It thus seems certain that the high estimates of mutation rate from the numbers of resistant individuals are not the product of an upward bias, and we may now turn to the possibility that the low estimates from the numbers of resistant clones are due to a downward bias. Two alternative possibilities have been suggested which would account for these lower values: (1) a change to a low rate of mutation during the later part of growth, and (2) a delay in the phenotypic expression of a mutation.

The problem of distinguishing between these will be simplified if we consider the first and determine the time at which the supposed transition would have to occur, and whether this time is related to the number of generations from resting stage or to the approach to saturation.

To determine whether the supposed transition would be a function of the number of generations from resting stage the data of table 1 may be used. Half the cultures were inoculated with 10 and half with 10,000 bacteria, and

these passed through 25 and 15 generations respectively (the relevant data are given in table 5). The mutation rates calculated by methods 1 and 2

TABLE 5

Generations between resting stage and the approximate time of the first mutation in series of liquid cultures started from widely different inocula showing that mutation rate is independent of this variable over the range of 6 to 18 generations. (Data from table 1.)

EXPERIMENT	A	B	C	D	E	F	G	H
Inoculum (no. of bact.)	10	10	10	10	10^4	10^4	10^4	10^4
End no. of bact. $\times 10^8$	3.1	4.6	2.4	2.8	4.2	3.7	3.2	3.8
Generations growth	24.9	25.5	24.6	24.7	15.4	15.2	15.0	15.8
Max. resist. b. in series	447	221	158	79	242	376	725	263
Gen. after first mutation	8.8	7.8	7.3	6.4	8.0	8.6	9.5	8.1
Gen. to first mutation	16.1	17.7	17.3	18.3	7.4	6.6	5.5	7.7
Mut. rate (method 1), $\times 10^{-8}$	.42	.28	.59	.53	.31	.34	.40	.33
Mut. rate (method 2), $\times 10^{-8}$	5.1	1.5	3.8	2.1	2.1	4.5	5.8	3.9

showed no effect due to the difference in number of generations from resting stage. Thus the time of the supposed transitions would have to be a function of the approach to saturation, and not of the number of generations from resting stage. This means that mutation rate would have to be of the order of 3×10^{-8} during the whole of the logarithmic growth phase, dropping to something like $.4\times10^{-8}$ during the last few divisions.

It will be shown later that there are apparent variations in mutation rate during early growth, but that they are associated with the first few divisions after resting stage and occur too early to have any bearing on the immediate problem.

It is thus evident that the possible effect of a low mutation rate during the later part of growth can be eliminated by confining one's tests to the period of rapid growth; and critical evidence for or against a possible delay can be obtained from estimates of mutation rate based on the number of resistant clones appearing during rapid growth.

EVIDENCE FOR A DELAY BETWEEN MUTATION AND PHENOTYPIC EXPRESSION (METHOD 4)

It now remains to determine the rate of appearance of resistant clones during rapid growth, when no approach to saturation is involved.

If the rate of appearance of resistant clones is high (3×10^{-8}), the discrepancy between methods 1 and 2 can be interpreted without assuming a delay between mutation and phenotypic expression. If, on the other hand, rate of appearance of resistant clones is low ($.4\times10^{-8}$), then from the previous evidence the number of individuals in an average resistant clone must be greater than expected on the basis of the number of generations passed through after

its first appearance. This would be interpreted as indicating that the mutant clone had its origin one or more divisions prior to its becoming phenotypically detectable.

One obvious alternative to this interpretation should be mentioned, namely that the excess numbers of mutants are due to more rapid division in these than in the parent strain. This is rendered unlikely however by the evidence of DEMEREC and FANO (1945) that these mutants do not divide more rapidly, and in addition more critical evidence against the possibility has been obtained from the experiments described under method 3.

The experimental procedure was essentially that used by DEMEREC (1946) in his work on mutation rates in *E. coli* following irradiation. Bacteria are grown on agar for varying periods of time, sprayed with phage, and incubated until colonies appear. Mutations occurring during growth, and gaining phenotypic expression, will give rise to resistant clones. Since individual bacteria cannot move about on the agar, the members of a clone are confined to a particular locality. These resistant clones survive the application of phage, and eventually form colonies of visible size. Thus each mutation which gives rise to phage resistance is in the end represented by one colony.

An estimate of mutation rate is obtained by dividing the number of resistant clones appearing in a given period by the number of bacterial divisions times 1/ln 2. Thus, if R_1 and R_2 are the numbers of resistant clones present at times 1 and 2, the number of resistant clones arising during the interval between times 1 and 2 is R_2-R_1. Similarly, if N_1 and N_2 are the numbers of bacteria present at times 1 and 2 respectively, the increase during the interval will be N_2-N_1. Since each division of a bacterium increases the total number by one, this value is equal to the number of bacterial divisions during the period. Mutation rate per bacterium per division cycle, a, will therefore be obtained from the formula:

$$a = (\ln 2)(R_2 - R_1)/(N_2 - N_1). \qquad (4)$$

Since the values of R_1, R_2, N_1 and N_2 represent viable cells only, an assumption is involved, namely that all bacteria divide. It will be shown later that this assumption is approximately correct for the early stages of logarithmic growth, and it is assumed that no appreciable increase in the proportion of cells which fail to divide, takes place until the phase of declining growth rate is approached. In these experiments precautions were taken to ensure that growth is limited to the period of exponential increase.

It will also be shown that the effect of the presence of cells which do not divide further, will be to increase the estimated rate of mutation. The present experiments can therefore be considered critical if the estimates of mutation rate obtained using method 4 are found to be low relative to those obtained using methods 2 and 3.

Estimates of the values of R_1, R_2, N_1, and N_2 are obtained as follows. Four plates are inoculated with a suitable number of bacteria, two being incubated until time 1 and two until time 2. One plate from each incubation period is sprayed with phage and then incubated further. The numbers of colonies

developing on these plates represent the numbers of resistant clones present at the time of spraying, that is, R_1 and R_2, respectively. The remaining plate from each incubation period is washed with ten cc of normal saline and the numbers of bacteria present (N_1 and N_2, respectively) determined by colony counts.

Where time 1 is the time of plating the bacteria, and no divisions can have taken place, N_1 is determined in a more direct manner. Instead of plating and then washing off the bacteria plated, an equivalent quantity of the culture from which the inoculum was taken is diluted and colony counts made.

An estimate of mutation rate is thus obtained from four plates. In all experiments these four plates were replicated several times, and a corresponding number of independent estimates of rate obtained. These independent estimates have been averaged, and the standard deviations calculated.

In all experiments in which growth was determined by the use of duplicate plates, care was taken to ensure the same amount of growth on both plates. All plates were warmed in the incubator before plating the bacteria, and when removed for plating were kept warm on a thermostatically controlled warm table until returned to the incubator. All platings and removals from the incubator followed an accurately timed schedule. The temperature in the incubator was kept as uniform as possible by circulating the air rapidly with fans. At the end of incubation, growth was stopped abruptly by chilling plates in contact with the cold metal of a refrigerator freezing unit. This chilling did not affect the survival of the bacteria or the phenotypic expression of the mutants.

Additional precautions were required with respect to (1) the choice of cultures from which to inoculate the plates, (2) the number of bacteria plated, and (3) the amount of phage applied by spraying.

(1) When relatively large numbers (of the order of 10^8) of bacteria are plated, there will be a certain number of resistant cells in the inoculum. These resistant cells have occurred by mutation during the growth of the culture from which the inoculum is taken. Cultures vary widely in the number of mutants present at the end of growth, and in these experiments the number present in the inoculum (R_1) was determined by spraying with phage immediately after the bacteria had been plated. Where the number is excessive it is apt to obscure the increase in resistant clones resulting from growth, or to render the determination of the increase less accurate. For this reason, cultures having an excessive number of resistant cells were not used as inocula. The cultures that were used contained from 5 to 50 resistant bacteria per 10^8 sensitive.

There is no evidence that this selection biased the results, since mutation rates obtained using these inocula were the same regardless of the number of resistant bacteria present.

(2) The size of the inoculum was adjusted so that the end number of bacteria on the plate would be approximately 2×10^9. With end numbers of less than this the number of resistant colonies was reduced, and at the same time the accuracy of the method. With excessively large end numbers of bacteria there is a reduction in the apparent mutation rate. The precise interpretation

of the phenomenon is uncertain. In the absence of evidence to the contrary it has been assumed that mutations do occur at the normal rate, but that they fail to develop visible colonies owing to the presence of large numbers of sensitive bacteria that are not lysed by the phage—a situation which occurs if phage is applied after bacterial growth has passed the logarithmic phase.

Tests showed that this apparent reduction in mutation rate occurred only if the end number of bacteria exceeded 5×10^9.

(3) It is known that bacteria which are infected during rapid growth have a latent period of 13 minutes—at the end of which time they burst, liberating on the average 180 phage particles (DELBRÜCK and LURIA 1942). If some of the bacteria fail to be infected at the time of spraying, it is unlikely that they will escape infection once lysis of the others starts. Any uninfected individuals would, on the average, pass through somewhat less than one division during the 13-minute latent period. Thus, with a large proportion of the bacteria uninfected at the time of spraying, a somewhat less than twofold increase in population would be expected before all the bacteria became infected, and the apparent mutation rate from such an experiment would be increased proportionally. Where all but a small proportion of the bacteria are infected at the time of spraying, the apparent mutation rate would not be appreciably greater than in the case of 100 percent infection.

In the present experiments the number of phage particles applied was equal to the number of bacteria, or slightly in excess. Large excesses were not used since these involve long periods of spraying, with resultant wetting of the surface of the agar and a tendency for the bacteria to be moved about by the moisture. Larger numbers of phage particles have been used, however, by BEALE (1948), who concentrated the phage by centrifuging. The estimates of mutation rate that he obtained in this manner do not differ appreciably from those obtained in the present experiments, and it may be assumed that the quantities used in the latter were adequate.

By the method described in this section it was possible to determine the rate with which resistant clones appear during the period of rapid growth from the resting stage onward.

The results of four separate experiments, each with eight independent replicates, are given in table 6. In these experiments the bacteria were grown over a period of approximately eight generations; and the mutation rate obtained is that for the whole growth period.

It will be seen that the average of estimates of rate from all experiments is low ($.59 \times 10^{-8}$) and is in close agreement with the low estimates from method 1 (average $.40 \times 10^{-8}$). As pointed out earlier, this constitutes evidence that resistant clones arise from mutations occurring one or more generations prior to their first becoming detectable.

It should be mentioned at this point that subsequent experiments, described in the next section and summarized in figure 1, have enabled mutation rate to be calculated from a tenfold increase in bacterial titer onward. This eliminates the contribution of the first few divisions, during which an excessive number of

TABLE 6

Mutation rate of B/r to resistance to phage T1, estimated from the number of resistant clones appearing during bacterial multiplication on agar (method 4 of this paper).

EXPERIMENT*	A	B	C	D
Inoculum (no. of bact.), $\times 10^8$	.115	.119	.106	.055
Resist. b. in inoc., $\times 10^{-8}$	30.2	30.2	10.0	38.0
Incr. in no. of bact.	264×	250×	345×	317×
Generations of growth	8.1	8.0	8.5	8.3
Replicates	Mutation Rate, $\times 10^{-8}$*			
1	.49	.54	.48	.53
2	.47	.61	.69	.38
3	.56	.70	.57	.49
4	.42	.49	.59	.71
5	.70	.54	.89	.50
6	.69	.46	.44	.52
7	.45	.51	.69	.63
8	.53	.66	1.04	.89
Av. mutation rate, $\times 10^{-8}$	.54	.57	.67	.58
Standard deviation	.098	.078	.205	.156
Mut. rate from 10× increase onward, $\times 10^{-8}$†	.45	.48	.60	.52

* Each replicate mutation rate is calculated from an independent single-plate estimate of each of the following four values: (1) number of bacteria in the inoculum, (2) end number of bacteria, (3) number of resistant bacteria in the inoculum, and (4) end number of resistant clones, using formula 4 of this paper.

† This mutation rate is calculated from a tenfold increase onward using the information in figure 1 and the method outlined in the section on method 4. It is a more accurate estimate of the rate of formation of resistant clones during logarithmic growth, since the bias from the high early rate of appearance of resistant clones is removed.

resistant clones appears, and reduces the average rate obtained from these experiments from $.59\times10^{-8}$ to $.51\times10^{-8}$.

RATE OF APPEARANCE OF RESISTANT MICROCOLONIES DURING THE EARLY DIVISIONS ON A SOLID MEDIUM

Where there is a delay between mutation and phenotypic expression such that the first phenotypically resistant individual appears n generations after the mutation has occurred, there will be 2^n bacteria in the mutant clone when it first becomes detectable. When a mutation occurs, a delay of n generations in the time of its appearance will correspond to a 2^n-fold increase in the population. The apparent number of bacterial divisions in which the mutation has occurred will then be 2^n times the true number, and when an estimate is made from the number of detectable mutant clones, the apparent rate will be reduced to $\frac{1}{2}^n$ of the true value.

From the interpretation here adopted, it follows that a sample from a culture will contain some mutant individuals which are not yet phenotypically resistant. When such a sample is spread on agar and allowed to grow (as in method 4) these hidden mutants develop phenotypic resistance during the first few divisions and thus give rise to resistant microcolonies. Since a mutant clone may contain a number of these hidden mutants, and since these are dispersed over the surface of the agar, a number of resistant microcolonies can

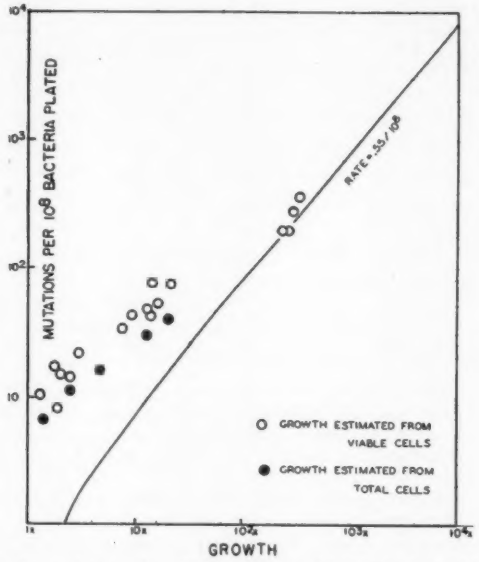

FIG. 1.—Numbers of mutations to phage (*T1*) resistance arising during bacterial multiplication, as estimated from the numbers of resistant microcolonies present after varying periods of growth.

result from a single mutation prior to plating. This is of course not true for mutations occurring after plating, because the products of these are confined to one locality.

Thus the rate of appearance of resistant microcolonies during the early divisions would be expected to be high, approaching the true mutation rate; and the rate of appearance during later divisions would be expected to be low, approaching 2^n times the true mutation rate.

Evidence has been obtained on this point, using method 4 and plotting the numbers of resistant clones arising during varying periods of incubation ($R_2 - R_1$, where time 1 is the time of plating) against growth in terms of factor increase in the number of bacteria (N_2/N_1). The absolute increase in resistant clones with growth depends, of course, upon the original number of bacteria plated, and where data are obtained from a number of separate experiments they are comparable only if expressed in terms of the number of individuals in

the inoculum. It is therefore convenient, when plotting a curve using data from a number of experiments, to express the increase in terms of resistant clones per 10^8 bacteria in the inoculum. This has been done in figure 1, where $(R_2-R_1)/N_1\times10^8$ is plotted against growth (N_2/N_1), time 1 being the time of plating. Each point shown in figure 1 was obtained by averaging at least eight independent estimates of increase in number of resistant clones and of growth.

Two experimental procedures for estimating growth were used in this case. One was that mentioned earlier, in which bacteria are washed from the plate and the number determined by dilution and assay. The other was by direct count of the numbers of bacteria in the developing microcolonies.

For the latter purpose small numbers of bacteria were plated on agar, and the plates were incubated at the same time and for the same period as those that were to be sprayed with phage. These growth assay plates were then chilled to stop division and examined under a high-power dry objective. The numbers of bacteria in 50 microcolonies were counted and averaged. The method gave accurate results up to an increase in number of bacteria of approximately 64 times.

In figure 1, points obtained by calculating growth by means of assay of bacteria washed from duplicate plates are shown as hollow dots. Those for which growth was calculated from the average number of bacteria per microcolony are shown as solid dots.

Something similar to the expected high early rate of appearance of resistant clones is observed in these results. In figure 1 the numbers of resistant clones appearing during growth are plotted on a logarithmic grid against growth expressed in terms of factor increase in numbers of bacteria. For comparison, a curve is drawn showing the expected numbers—assuming a rate of $.55\times10^{-8}$ and immediate phenotypic expression. The experimental curve does show a high early rate, declining as growth proceeds. This is qualitatively what would be expected where there is a delay, though the rate is actually higher than expected.

The increase in resistant clones when material is sprayed with phage after a twofold increase in population is approximately 12 per 10^8 bacteria plated, which represents an apparent mutation rate of 8.3×10^{-8}; whereas the rate expected during the first division is the same as that obtained from liquid cultures, that is, something between 2.8 and 3.4×10^{-8}.

Furthermore, by extrapolating the curve backward it would appear that about seven per 10^8 bacteria become resistant during the lag phase before any division has taken place.

This point has been studied by incubating bacteria with a known lag phase of 70 minutes for 30, 60, and 90 minutes on agar and then spraying with phage. The results are given in table 7, and show an appreciable increase in resistant colonies from plates sprayed just before the onset of the first division and during the very early part of the division.

The points just considered are not directly related to the main issue. The important contribution of these experiments is to show that the rate of appearance of resistant clones declines during the first few divisions on agar.

A METHOD OF REDUCING THE DOWNWARD BIAS IN METHOD 2 DUE TO THE DELAY IN PHENOTYPIC EXPRESSION (METHOD 5)

As mentioned in the introduction, a delay between mutation and phenotypic expression must be variable within a mutant clone, expression occurring in some lines of descent earlier than in others. This leaves open the questions of the extent of the delay, the nature of the variation, and the rate of gene muta-

TABLE 7

Increase in number of bacteria resistant to phage T1 during early growth, using strain B/r grown on agar, and spraying with phage during the lag phase and early part of the first division.

EXPERIMENT	A	B	C
Replicate plates	10	10	10
Bact. in inoculum, $\times 10^8$	11.0	11.0	11.0
Resist. bact. in inoc., per 10^8	10.1	10.1	10.1
Incubation, minutes	30	60	90
Incr. in no. of bact.	1.0×	1.0×	1.3×
New resist. bact., per 10^8	0.1	5.8	10.7
Standard deviation	1.89	1.87	2.05

tion, the last being to a greater or smaller degree underestimated by the methods dealt with so far. Method 5 is designed to obtain a less biased estimate of mutation rate, and, from this, some idea of the extent of the delay.

With a variable delay it would be expected that mutant clones arising from mutations early in the growth of a culture would contain a higher proportion of phenotypically resistant cells than would younger mutant clones, and that a higher and less biased estimate of mutation rate would be obtained if it could be calculated from these older clones alone.

To do this the following method has been devised. In a series of similar test cultures a few of the cultures contain many times the average number of resistant cells, because of the chance occurrence in these cultures of an early mutation. In such cases the precise numbers of resistant bacteria from the earliest mutation and from subsequent mutations cannot be determined directly. The probable number from later mutations, however, is approximately equal to the mean number in the whole series. This is, incidentally, a very slight overestimate, and a more precise approach will be considered later.

Using this method, the probable number of resistant bacteria descended from the earliest mutation in a series of cultures is obtained by subtracting the mean number of resistant bacteria per culture, r, from the highest number occurring in any one of the cultures, h. At the probable time of occurrence of this mutation the population in the culture would have been $N/(h-r)$, where N is the end population; and the population in the whole series of cultures would have been $CN/(h-r)$, where C is the number of cultures in the series. Thus the first mutation in the series occurred when the population was $CN/(h-r)$, and since the inocula were small the number of bacterial divisions

giving rise to this population would also be $CN/(h-r)$. The mutation rate, a, when one mutation occurs in this number of bacterial divisions is of course given by the formula:

$$a = (\ln 2)(h-r)/CN. \qquad (5)$$

Mutation rate is very slightly underestimated by this formula, since r is an overcorrection for the probable number of resistant cells from mutations subsequent to the first. A more precise correction would be obtained by averaging the number of resistant cells per culture, exclusive of those from the first mutation in the series. This complication has not been introduced, however, since the gain in accuracy would not be appreciable.

The rates calculated in this manner may be considered as approaching the true rate if most of the members of these older clones have become phenotypically resistant. If only a small proportion have become resistant, this rate is still an underestimate, although it would be closer to the true rate of gene mutation than the estimates using method 2. This method will be known as method 5.

The rates obtained in this manner, using the data from table 1, are presented in table 8, together with the values required for the calculations. Data of

TABLE 8

Mutation rate of B/r to resistance to phage T1, calculated from the maximum number of resistant bacteria in any one culture of a series, using method 5 of this paper, and the data in table 1.

EXPERIMENT	A	B	C	D	E	F	G	H
Number of cultures	25	25	25	25	25	25	25	25
Bact. per cult., $\times 10^8$	3.1	4.6	2.5	2.8	4.2	3.7	3.2	3.8
Av. resist. b. per cult.	56.1	18.5	29.5	16.9	26.8	59.5	68.6	52.6
Max. no. resist. bact.	447	221	158	79	242	287	725	263
Mutation rate, $\times 10^{-8}$	3.6	1.3	1.3	.83	1.5	2.3	5.7	1.5

LURIA and DELBRÜCK, and DEMEREC and FANO, obtained using phage *T1*, have been treated similarly, and a detailed comparison is made in table 9 of rates from methods 1, 2, and 5, using information from all sources. For the sake of convenience, the rates obtained by methods 2 and 5 have been expressed in the last two columns of the table in terms of the smaller value from method 1, as ratios—rate (2)/rate (1), and rate (5)/rate (1).

Taking into consideration all available information on mutation to resistance to phage *T1*, rates from methods 2 and 5 differ only slightly and are between four and nine times the rates from method 1.

The application of method 5 has also been extended to the data obtained by DEMEREC and FANO using phages other than *T1;* and averages of mutation rates from methods 1, 2, and 5, and of the ratios rate (2)/rate (1), and rate (5)/rate (1), have been worked out for all available data on strains *B* and *B/r* and phages *T1*, *T3*, *T4*, *T5*, *T6*, and *T7*. They are given in table 9.

The purpose of these calculations was to determine whether mutation rate when estimated from the older mutant clones only is higher than when estimated from the average numbers of resistant bacteria per culture. If the delay is sufficiently variable and of sufficient magnitude, it would be expected that

TABLE 9

Mutation rates of strains B and B/r to resistance to phage T1, from series of similar liquid cultures started with small inocula, using methods 1, 2, and 5 and calculating from data of LURIA *and* DELBRÜCK *(1943, table 3, experiment 23),* DEMEREC *and* FANO *(1945, table 4), and the present paper (tables 1 and 8). All the experiments which are suitable for the application of the three methods have been used.*

EXPER.	STRAIN	CULTURES	RATE (1) $\times 10^{-8}$	RATE (2) $\times 10^{-8}$	RATE (5) $\times 10^{-8}$	RATIO (2)/(1)	RATIO (5)/(1)
LURIA and DELBRÜCK							
A	*B*	87	.32	2.4	1.5	7.5	4.4
DEMEREC and FANO							
A	*B*	53	.63	1.4	1.8	2.2	2.9
B		53	.33	2.3	6.1	7.0	18.5
C		17	1.15	3.9	9.2	3.4	7.9
D		25	.71	3.1	4.1	4.4	5.8
E		19	.66	3.2	3.0	4.8	4.5
F		38	1.26	6.1	7.8	4.7	6.2
G		45	.24	0.8	1.3	3.3	5.4
H		44	.63	3.2	6.0	5.7	9.6
I		45	.31	2.0	2.9	6.4	9.4
J		45	.92	1.3	1.3	1.4	1.4
		Average	.68	2.7	4.4	4.3	7.2
Present investigation							
A	*B/r*	25	.42	5.1	3.6	12.2	8.6
B		25	.28	1.5	1.3	5.4	4.4
C		25	.59	3.8	1.3	6.6	2.1
D		25	.53	2.1	8.3	4.0	1.6
E		25	.31	2.1	1.5	6.8	4.7
F		25	.34	4.5	2.3	13.2	6.7
G		25	.40	5.8	5.7	14.5	13.8
H		25	.33	3.9	1.5	11.8	4.6
		Average	.40	3.6	2.3	9.3	5.8

in these older clones a higher proportion of the mutants would have become phenotypically resistant.

Not all experiments show a difference between estimates of rate from the average resistant bacteria (method 2) and estimates from the highest number of resistant bacteria, of which the majority are presumably descended from an early mutation (method 5). Examining data from all sources, the estimates from method 5 and from method 2 do not differ significantly.

With the exception of the mutations to *B/3, 4* and *B/3, 4, 7*, estimates from method 5 are of the order of six to seven times those from method 1, and the lower ratios in the case of these exceptions are in all probability due to the high proportion of slow-growing *B/3, 4*, and *B/3, 4, 7* mutants—DEMEREC and FANO having shown in their experiments on competitive growth that these mutants grow much more slowly than the parent strain *B*.

A crude average of the ratio rate (5)/rate (1) from mutations other than those to *B/3, 4*, and *B/3, 4, 7* shows that estimates obtained by method 5

TABLE 10

Mean mutation rates of strains B and B/r to phage resistance, estimated by methods 1, 2, and 5; data of LURIA *and* DELBRÜCK, DEMEREC *and* FANO, *and the present investigation (table 9), all from series of similar liquid cultures started with small inocula.*

PHAGE	STRAIN	RESISTANT BACTERIA	NO. OF EXPER. AVERAGED	AVER. RATE (1) $\times 10^{-8}$	AVER. RATE (2) $\times 10^{-8}$	AVER. RATE (5) $\times 10^{-8}$	AVER. RATIO (2)/(1)	AVER. RATIO (5)/(1)	SOURCE
T1	*B/r*	*B/r/1,5; B/r/1*	8	.40	3.6	2.2	9.3	5.8	table 9
T1	*B*	*B/1,5; B/1*	1	.32	2.4	1.5	7.5	4.4	L. & D.
T1	*B*	*B/1,5; B/1*	10	.68	2.7	4.4	4.3	7.1	D. & F.
T5	*B*	*B/1,5*	5*	.38	1.5	2.6	4.3	7.5	D. & F.
T6	*B*	*B/6*	3	5.0	30.8	42.0	5.3	7.1	D. & F.
T3	*B*	*B/3,4,7; B/3,4*	7	5.1	10.0	12.5	2.3	2.9	D. & F.
T4	*B*	*B/3,4,7; B/3,4*	3	5.5	12.9	34.8	2.7	5.8	D. & F.
T7	*B*	*B/3,4,7*	3	4.4	12.2	9.6	2.8	2.4	D. & F.

* Note: One experiment containing an exceptional culture with 20,000 resistant bacteria has been omitted from these averages.

are approximately 6.4 times those obtained by method 1. The significance of this ratio in relation to the extent and variability of the delay between mutation and phenotypic expression will be considered in the discussion.

THE INFLUENCE OF DIVISION MORTALITY UPON ESTIMATES OF MUTATION RATE

All the methods of estimating mutation rate are subject to a small error because some of the bacteria resulting from a division fail to divide again (WILSON 1922). The number of mutations occurring during a division is thus underestimated, and the number of divisions, where calculated from numbers of viable bacteria, is also underestimated.

It might be assumed that the apparent variations in the mutation rate during the early part of the growth cycle are due to corresponding changes in the mortality associated with division. That this is not the case will be demonstrated; and corrections to be applied to mutation rates which have been based upon the assumption of unity survival will be considered.

Where growth has been estimated from the numbers of *viable* bacteria, a correction may be obtained in the following manner. Let S be the chance of

survival of one of the bacteria resulting from a division. In a population of N viable bacteria, one division will raise the number from N to 2NS viable individuals, and, where A is the true rate of mutation, will result in the production of ANS/ln 2 viable mutant individuals. If the increase in population is determined from assays of viable bacteria, the apparent mutation rate, a, during this division will be (ln 2) (ANS)/(ln 2) (2NS−N), which reduces to AS/(2S−1). The true mutation rate may thus be obtained from the apparent rate by the formula:

$$A = a(2S - 1)/S. \tag{6}$$

The relationship between the true mutation rate and an apparent rate, a_1, obtained by utilizing the increase in *total* population as distinct from viable population, may be derived in a similar manner and is expressed by the formula:

$$A = a_1/S. \tag{7}$$

Where estimates of mutation rate have been made in both ways, that is, using both viable population increase and total population increase, the value of S may be determined from the apparent mutation rates a and a_1. The formula for this as obtained from formulas (6) and (7) by simultaneous solution and is:

$$S = (a + a_1)/2a. \tag{8}$$

The true mutation rate also may be obtained from the values a and a_1, using equation (8) and substituting for S. This yields the formula:

$$A = 2a \cdot a_1(a + a_1). \tag{9}$$

These formulae may be applied to the data from figure 1 in order to determine whether the high apparent mutation rate during the first few divisions could be due to a low survival. It will be noted that points on figure 1 obtained by estimating growth from viable numbers of bacteria do not differ greatly from those obtained by estimating growth from total bacteria. This suggests that mortality due to division is not excessive, and we may calculate the chance of survival of the individual products of one division, by using formula (8). Thus, over the period from a twofold to a tenfold increase in population, the rates estimated from the two curves would be approximately 2.6×10^{-8} and 1.4×10^{-8}, respectively. From these two values the calculated survival is 0.75. Therefore the high rate of appearance of resistant clones during the early divisions on a solid medium discussed earlier cannot be due to an excessive division mortality.

It should also be noted that the estimate of rate which would have been obtained over this period of growth by either method, had survival been unity, is given by formula (9) and is 1.9×10^{-8}. This latter figure, however, has little bearing on the present discussion, as it is in any case intermediate between the estimates of rate obtained from numbers of resistant clones and from numbers of resistant individuals.

It is realized that this early low division mortality may not persist throughout the whole of the growth cycle but may rise as the population in a culture approaches the saturation density. In order to minimize the possibility of this effect, the end population of cultures grown on agar (method 4) was not allowed to reach that period at which growth rate begins to decline (approximately 5×10^9 bacteria on a 100 millimeter plate of broth agar). If, despite these precautions, there was an increase in division mortality, then as pointed out earlier, the estimates of mutation rate obtained would be somewhat greater than the true rate. It will be noted that this would tend to weight the data obtained by method 4 against the interpretation arrived at. We may therefore conclude that errors from this source do not affect the main conclusions.

DISCUSSION

From the experiments described, it is apparent that those methods which take into account only the number of resistant clones developing in a given period, and not the number of resistant individuals in these clones, give a low estimate of the mutation rate (see table 11 for summary).

Method 1 falls in this category, since it is based upon the proportion of cultures in which no phenotypic mutants have appeared—this being a function of the total number of phenotypically resistant clones in the series of cultures—and is independent of the size of the clone.

Method 4 also falls in this category, since the members of a mutant clone are confined to one spot on the agar and after one member has become resistant others may change in the same manner without being detected. Method 4, which uses the number of resistant clones appearing during rapid growth, provides a necessary check on method 1, which uses the number of resistant clones developing during growth to saturation, and which would be strongly biased if resistant clones appeared at a different rate during the few divisions prior to the cessation of growth. The estimates of rate obtained by these two methods are, within the limits of experimental error, similar, and in the present experiments—using resistance of strain *B/r* to the phage *T1*—average approximately 0.5×10^{-8}.

Methods that are based on the increase in numbers of resistant individuals developing during growth, however, as distinct from the numbers of resistant clones, produce much higher estimates of mutation rate. The following fall in this category: method 2, which uses the average number of resistant bacteria in a series of similar cultures started with small inocula; method 3, which uses the number of resistant bacteria in a single culture started with a large inoculum; and method 5, which uses the highest number of resistant bacteria in any one culture of a series.

Method 3, which is based on a relatively direct calculation, provides a necessary check on method 2, which is subject to a statistical bias. Similarly, method 5, which is based on the early mutations, provides a check on method 2, which would be biased if the delay in developing phenotypic resistance were excessive in an appreciable proportion of the lines of descent within a mutant clone. Method 3 is also of use in this connection in the one experiment in which

TABLE 11

Averages of mutation rates for strains B and B/r to resistance to phage T1 obtained by methods 1 to 5. Summary of calculations using data, or mutation rates already calculated, from the present investigation, LURIA *and* DELBRÜCK *(1943),* DEMEREC *and* FANO *(1945), and* BEALE *(1948).*

METHOD	FORMULA	MUTATION RATE, $\times 10^{-8}$ Present	L. & D.	D. & F.	BEALE
Estimates based on numbers of resistant clones					
(1) Series of liquid cult., no resist. b.	$a = -(\ln 2)(\ln P_0)/N$	0.40	0.32	0.68	—
(4) Solid medium, resist. clones	$a = (\ln 2)(R_2 - R_1)/(N_2 - N_1)$	0.51	—	—	0.49*
Estimates based on numbers of resistant individuals					
(2) Series of liquid cult., av. res. b.	$r = (aN/\ln 2)\ln(CaN/\ln 2)$	3.6	2.4	2.7	—
(3) Liquid cult. from large inocula	$a = (\ln 2)(r_2/N_2 - r_1/N_1)/g$	3.1	—	—	—
(5) Series of liquid cult., max. res. b.	$a = (\ln 2)(h - r)/CN$	2.2	1.5	4.4	—
Early growth					
(4a) Solid med., res. clones, first div.	$a = (\ln 2)(R_2 - R_1)/(N_2 - N_1)$	8.3	—	—	—

Symbols:

a, mutation rate per bacterium per division cycle.
r, average number of resistant bacteria per culture.
h, highest number of resistant bacteria in any one culture of a series.
N, average number of bacteria per culture; N_1 and N_2, average number at times 1 and 2 respectively.
C, number of cultures.
R, average number of resistant clones per plate; R_1 and R_2, average numbers at times 1 and 2 respectively.
g, number of cell generations.
P_0, proportion of test cultures in which there are no resistant mutants.

* Note: the value $.7 \times 10^{-8}$ bacterial divisions obtained by BEALE has been converted to the rate per bacterium per division cycle by multiplying by ln 2 (=.693).

the growth period is long, and in which the majority of the resistant individuals are from mutations occurring many generations previous to the cessation of growth.

The estimates of rate obtained by these methods are all considerably greater than those which took into consideration only the numbers of resistant clones and not the numbers of resistant individuals, and differ only slightly among themselves. In the present experiments they average approximately 3×10^{-8}.

It is thus evident that the numbers of resistant individuals in a mutant clone must be greater than would be expected on the basis of the time of phenotypic appearance of that clone. One simple interpretation of this—namely,

that the growth rate is higher in the mutant clones than in the parent strain—is ruled out by the results obtained using method 3. The only plausible interpretation, therefore, seems to be that a mutation to resistance is not phenotypically expressed until after a number of divisions have taken place.

Two possibilities exist with regard to such a delay: (1) that after a fixed period of one or more generations all of the offspring of the original mutant become phenotypically resistant at the same time, and (2) that the delay is variable within the mutant clone, some lines of descent becoming resistant earlier than others. As pointed out by LURIA (1946), the presence of cultures having only one resistant bacterium eliminates the first of these. Thus the delay must be variable within the mutant clones, and a single individual may develop phenotypic resistance in the first instance, being followed later by other members of the clone.

As pointed out, the presence of phenotypically susceptible individuals in the younger mutant clones, and the occurrence of changes in these to phenotypic resistance, results in a high apparent mutation rate when the members of the clone are dispersed and the changes are thus rendered detectable in each individual, and in a low apparent mutation rate when the members of a clone are grouped together and only the first change to phenotypic resistance is detectable. In the early growth on agar a situation exists in which the members of mutant clones in the inoculum are dispersed over the plate but the products of subsequent divisions of individual bacteria are grouped together. In such circumstances the apparent mutation rate would be expected to be high during the first division, as in liquid cultures, and to decline during subsequent generations to the apparent rate obtained when only the numbers of resistant clones are considered. This, in fact, has been observed and lends support to the conclusions reached.

In this connection, however, it should be noted that the rate of appearance of resistant bacteria during the first division on agar following a resting stage is higher than expected, by a factor of approximately three or four. This does not affect the main line of reasoning, although it is of interest in itself and appears to be well substantiated by the data.

Attempts to interpret this as due to the bacteria's failing to adsorb phage during the early stages of growth after a resting stage have not been successful, since one would have to suppose complete failure of adsorption over a period of at least two generations.

Two possibilities remain: (1) that of a genuine high rate of mutation during this division, together with physiological conditions favoring immediate phenotypic expression, since otherwise the products of these mutations would not be detectable; and (2) that of physiological conditions favoring phenotypic expression of mutants which had not hitherto become resistant. So far, no methods have been devised to distinguish between these two possibilities.

Thus the rate of appearance of resistant clones during early growth on agar is in agreement with the concept of a delayed phenotypic expression, although an additional phenomenon appears to be involved.

The size of a mutant clone at the time of appearance of the first resistant in-

dividual may be obtained by comparing the estimates of mutation rate from method 1, utilizing the number of resistant clones developing in liquid cultures, with those from method 5, utilizing the number of resistant individuals in old mutant clones. Table 9 may be used for this purpose, and the ratio rate (5)/rate (1) considered. If one omits the data on mutations to *B/3, 4* and *B/3, 4, 7*, because of the bias resulting from the known low growth rate of these mutants (DEMEREC and FANO 1945, table 7), the ratios in the cases of the remaining mutations appear to be similar. A crude average of these is 6.4. This indicates that there are approximately six or seven individuals in a mutant clone at the time of appearance of the first resistant individual; that is, that the delay between mutation and phenotypic expression in at least one member of the resulting clone is of the order of 2.7 generations.

The extent of the delay in individual lines of descent within a mutant clone other than the first one to become resistant, cannot be determined directly. However, the absence of any striking difference between the estimates of mutation rate obtained by methods 2 and 5 suggests that the delay is not excessive; and the similar values, 2.8×10^{-8} and 3.4×10^{-8}, obtained by method 3 with growth periods of six and eleven generations respectively, indicate that no very large proportion of a mutant clone changes to phenotypic resistance after six generations from the time of the mutation.

The possibility that the delay in expression of irradiation-induced changes is of the same origin as that observed in spontaneous mutations should be considered at this point, although a positive answer cannot be given, since strictly comparable studies of the induced changes have not yet been carried out, and, in particular, nothing is known of the numbers of resistant individuals developing in the late-appearing resistant clones resulting from irradiation.

If irradiation results in the immediate induction of gene changes similar to those occurring spontaneously, and the delay is one of phenotypic expression similar to that in untreated material, then the numbers of resistant individuals in the late-appearing resistant clones should rise rapidly after the clones first become detectable, because of susceptible members becoming phenotypically resistant, and should eventually approach the numbers in the resistant clones that appeared soon after treatment. Variation in the time of occurrence of phenotypic expression in different lines of the same mutant clone would also be expected in irradiated material.

Until information of this nature is obtained it is not possible to say with certainty that the observed delays in appearance of induced and spontaneous mutations are of the same origin. In the absence of more detailed information, one can only speculate on the basis of the variation between clones. Since in untreated material there is no striking difference between the apparent mutation rates as determined over periods of six and eleven generations (method 3), it would seem that six generations is adequate both for phenotypic expression in most of the mutants within a clone and also for expression in at least one individual in most mutant clones. In irradiated material an appreciable proportion of the resistant clones do not appear until after the sixth generation;

and it is therefore tentatively suggested that the two delays are not of the same origin.

It should also be noted that a delay in phenotypic expression has been observed in Paramecium following hybridization (KIMBALL 1937; SONNEBORN 1943; SONNEBORN and LYNCH 1934) and has been termed "cytoplasmic lag." This phenomenon is at least superficially similar to that described in the present work, in that "phenotypic expression . . . commonly appears first at slightly different times in different lines of descent within the clone" (SONNEBORN 1947). The extent of the delay appears to vary in Paramecium with the nature of the change, and in two instances of alteration in type there is no detectable delay. (These are the alterations from mating type I to mating type II in variety 1, and from mating type V to mating type VI in variety 3. For a discussion of this, see SONNEBORN 1947.) It will therefore be of interest to determine whether a similar diversity in the delay exists in bacteria, since the apparent differences in the mutations so far studied, all of which mutations are to resistance to one or another of the phages, are relatively small and can be explained as due to known differences in the growth rates of the mutants. Until more information of this nature is available it is not profitable to speculate further about whether "cytoplasmic lag" in protozoa and "delayed phenotypic expression" in bacteria have a common basis.

CONCLUSIONS

Evidence of a delay in the phenotypic expression of spontaneous bacterial mutations to phage resistance, using *E. coli*, strain *B/r*, has been obtained. This evidence comes from a comparison of the rate of appearance of phenotypically resistant clones during bacterial multiplication with the rate of gene mutation, the latter being estimated from the numbers of resistant individuals arising during similar periods of growth.

The rate of appearance of resistant clones per bacterium per division cycle is approximately 0.5×10^{-8}, whereas the numbers of resistant individuals correspond to a much higher rate of mutation, a rate of approximately 3×10^{-8}.

Since it is known that the mutants do not divide more rapidly than the parent strain, the excess number of individuals composing a mutant clone indicates that the clone was formed sometime prior to its becoming phenotypically detectable. Thus there appears to be a delay between mutation and phenotypic expression.

The possibilities of alternative interpretations of the data of previous workers on the basis of a statistical bias in one of the methods, and of a change in the mutation rate during the later part of growth, have been eliminated by using methods which are not susceptible to these sources of error.

A delay in phenotypic expression would be expected to result in a high rate of appearance of resistant clones during the first few divisions on agar, declining to the value 0.5×10^{-8} with further growth. This has been observed, although an additional phenomenon appears to be present which results in the appearance of a higher-than-expected number of resistant clones during the

first division. This phenomenon, although of interest in itself, does not affect the main conclusions.

It is estimated that phenotypic expression occurs in at least one member of a mutant clone approximately two or three generations after the mutation, and that in many cases it affects at first only one individual, others of the mutants becoming resistant during subsequent divisions.

Expression does not appear to be delayed in any appreciable proportion of a clone beyond approximately six generations after the mutation.

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ALTERNATIVE HYPOTHESES OF HYBRID VIGOR

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HYBRID vigor has been observed for centuries but explanations in terms of Mendelian heredity have, of course, been formulated only recently. The word heterosis was proposed by SHULL (1914) for this increase in vigor following the union of dissimilar gametes and has come into general use. SHULL (1908, 1911) and EAST (1908) believed that there exists a stimulus on crossing due to the genetic difference in the two germ plasms which would increase with the amount of difference. The alternative dominance hypothesis, first stated explicitly by BRUCE (1910) and KEEBLE and PELLEW (1910), has come to be widely accepted. This idea depends on the observation that there is a positive correlation between recessiveness and detrimental effect (or dominance and beneficial effect). According to this hypothesis some of the detrimental recessives brought into the hybrid zygote by one parent are rendered ineffective by their dominant alleles from the other. The result is an increase in vigor of the hybrid as compared with the parent stocks. Early objections to the theory were largely removed when JONES (1917) showed that with linkage and COLLINS (1921) showed that with a large number of factors, even in the absence of linkage, the consequences of the dominance hypothesis and the stimulation of heterozygosis hypothesis of EAST and SHULL were very similar and could not be distinguished in practice.

The purpose of this inquiry is to determine the maximum vigor that might occur under the conditions implied by the dominance hypothesis and to see if this theory provides an adequate explanation for observed increases in vigor on hybridization.

ASSUMPTIONS OF THE DOMINANCE HYPOTHESIS

For the purpose of this discussion it will be assumed that all genes concerned with vigor are completely dominant and that in each case the dominant allele is advantageous while the recessive is deleterious from the standpoint of survival. It will be assumed further that there are no complex interactions among these genes; they are either additive or multiplicative in their effect and each acts independently of the others. Crossing over will be assumed to be occurring freely so that there is no tendency for balanced heterozygotes to accumulate in the population due to reduced recombination.

These assumptions reduce the dominance hypothesis to its simplest form. An individual of maximum vigor would be one in which all gene loci contain

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at least one dominant factor. The difference in vigor between any individual and its theoretical maximum would be determined by the number of homozygous recessive loci. The maximum increase in vigor after hybridization would occur if each parent could supply all the dominant alleles lacking in the other and the hybrid were thus to receive at least one dominant gene at each locus. It would be possible to compute the maximum effect under this hypothesis by determining the increase in vigor that would result from the replacement with dominants of all homozygous recessive loci.

Another important assumption is that hybrid vigor is measurable in terms of selective value. This assumption is implicit in many of the discussions of theories of hybrid vigor, but it should be stated explicitly. The frequency of a detrimental recessive factor in a population is determined by its selective disadvantage, mutation rates, migration, and the size and breeding structure of the population. To whatever extent vigor is reflected by increased selective value or fitness, data concerning gene frequencies and selective values are useful in problems of hybrid vigor. It will be assumed here that increased vigor results in, and can be measured in terms of, selective advantage and the discussion is relevant only to vigor as defined in these terms, though the selection may, of course, be natural or artificial.

The frequency of detrimental recessives and their effect on the vigor of the population will now be considered, first for a large randomly mating population and later under other conditions.

LARGE RANDOMLY MATING POPULATIONS

Consider a large population mating at random in which the individuals homozygous for the recessive factor have a selective disadvantage of s as compared with the dominant phenotype. That is to say that the dominant and recessive phenotypes are surviving and reproducing in the ratio of 1 to 1-s. If the proportion of dominant factor *A* is p and the proportion of recessive factor *a* is q, or 1-p, the zygotic frequencies at equilibrium are given by the HARDY–WEINBERG rule. The terminology used here is essentially that of WRIGHT (1931, 1937, 1942).

Genotype	Frequency	Relative Selective Value
AA	p^2	1
Aa	2pq	1
aa	q^2	1-s

If mutation is occurring from *A* to *a* at a rate u per generation, the frequency of gene *A* will be reduced in the next generation by the quantity pu and the frequency of gene *a* will be increased by the same amount. In a given generation the ratio of the frequency of gene *A* to that of gene *a* is p/q. In the following generation, due to the effect of selection and mutation, this ratio becomes $(p^2 + pq - up)/[pq + q^2(1-s) + up]$. When the population reaches a state of equilibrium, the gene frequency ratio does not change from generation to generation. This may be stated algebraically as

$$\frac{p^2 + pq - up}{pq + q^2(1 - s) + up} = \frac{p}{q}.$$

The solution of this equation is $q^2 = u/s$, giving the recessive gene frequency, $\sqrt{u/s}$, obtained for this case by HALDANE (1927) and WRIGHT (1931). Usually u is much smaller than s; hence the frequency of the recessive gene *a* is small. As long as mutation rates are small compared with the selection coefficient, mutation in the same direction as selection pressure makes only a very slight change in the equilibrium point. Hence reverse mutation may be ignored with negligible loss in accuracy.

By the HARDY–WEINBERG rule, the proportion of homozygous recessive zygotes is q^2 or u/s, and each of these individuals has a selective disadvantage of s. The average reduction in selective value in the population due to this factor will be the product of the selective disadvantage of the factor and the proportion of individuals possessing the factor, which in this case is (s) (u/s) or u. Hence the average reduction in selective value due to a detrimental recessive factor is equal simply to the rate of mutation to that factor and is independent of its selective value, as has been pointed out by HALDANE (1937). The average effect on the population of a mildly deleterious recessive factor is the same as that of a strongly detrimental or lethal factor if they have the same mutation rate, the latter being eliminated more rapidly from the population and hence not affecting so many individuals. This is very convenient from the standpoint of analysis since average mutation rates are known much more accurately than average selective values.

If there are several loci in the genome capable of producing deleterious recessives, the total effect on the selective value of the organism will be the sum of the individual effects provided the gene effects are additive. If the number of such loci is n, and if $\bar{u}$ is considered as the average mutation rate for these n loci, the reduction in selective value due to homozygous deterimental recessives at all loci in which they occur is $n\bar{u}$. This is also approximately correct if the factors are multiplicative provided the individual gene effects are small.

The amount of increase in vigor, as measured by selective advantage, that would result if all the homozygous recessive loci were replaced with dominants would be $n\bar{u}$. Since n is the number of mutating genes in a haploid set of chromosomes, its value in Drosophila has been estimated by various methods. The maximum estimate would place the haploid number of gene loci at about 5000. It is not likely that the number is larger in other forms. The average mutation rate is probably less than 10^{-5}, so the product $n\bar{u}$ is not likely to be larger than .05.

If only lethals and semi-lethals are considered, the data from Drosophila provide more accurate estimate of the product of n and $\bar{u}$ than for either factor alone. According to DOBZHANSKY and WRIGHT (1941) who worked with the third chromosome of *D. pseudoobscura*, about three lethal or semi-lethal mutations occur per thousand chromosomes per generation. Assuming that the third chromosome makes up approximately one fifth of the total genome, the product $n\bar{u}$ for all lethal-producing loci in this species is about .015.

Data from *D. melanogaster* yield comparable results (MULLER 1928). Since there are certainly deleterious recessive mutations being produced which are not lethal or semi-lethal, these values must be considered as underestimates.

The milder mutants have been estimated by TIMOFEEFF-RESSOVSKY (1935) to be about twice as frequent as the more drastic. Considering all types of detrimental recessives, the value of nū is probably not larger than .05 as indicated previously.

If these estimates are correct, the selective advantage that would accrue to members of a population if all homozygous recessive factors were replaced would be perhaps 5 percent. This could be considered to be the greatest improvement in vigor, as measured in terms of selective advantage, that could occur due to hybridization. If only part of the recessive factors brought into the hybrid from one parent were covered by dominants from the other the improvement would be proportionately less. This means that the dominance hypothesis cannot, under the conditions postulated, account for increases of more than a few percent in vigor.

The above calculations require some modification if sex linked factors are involved, though the modification does not change the quantitative conclusions much. HALDANE (1937) has shown that X-chromosomal detrimental mutations are responsible for a loss of fitness of 3/2 u per locus, rather than u as in the case of autosomal recessives. However, virtually the entire effect is in the males (where this is the heterogametic sex) and the loss of fitness in females is negligible. This being the case and since there is no change in the number of exposed recessives in the male after hybridization, the X-chromosome has practically no effect on hybrid vigor. The calculations of maximum increase in vigor should be made on the basis of just the autosomes in those forms where sex chromosomes make up an appreciable portion of the chromatin material. Thus the estimates of possible hybrid vigor in Drosophila, for example, should be reduced somewhat.

So far, the only type of breeding population that has been considered is large, mating at random, and has been in this state long enough to be somewhere near equilibrium conditions. It is necessary to examine the situation when these conditions do not hold.

EQUILIBRIUM FREQUENCIES WITH INBREEDING

If the population is not mating at random but there is a specified amount of inbreeding, the conclusions still hold as may be shown for the case of close inbreeding leading to eventual complete homozygosity. Let the genotypes *AA*, *Aa*, and *aa* be in the proportions P, Q, and R respectively, the *aa* genotype have a selective disadvantage of s, mutation be occurring at the rate u from A to *a*, and the proportion, h, of heterozygosity be lost through inbreeding each generation. Reverse mutation again will be ignored.

Genotype	*A*	*Aa*	*aa*
Frequency	P	Q	R
Relative selective value	1	1	1−s

Increase due to mutation of *AA* genotypes	$-(2u-u^2)P$	$2u(1-u)P$	u^2P
Increase due to mutation of *Aa* genotypes		$-uQ$	uQ
Increase due to inbreeding	$\frac{h}{2}Q$	$-hQ$	$\frac{h}{2}Q$

The condition for equilibrium may be expressed by stating algebraically that the frequencies do not change from one generation to another. This leads to the equations.

$$\frac{R(1-s)+uQ+u^2P+hQ/2}{P+Q+R(1-s)}=R$$

$$\frac{P-(2u-u^2)P+hQ/2}{P+Q+R(1-s)}=P.$$

Noting that $P+Q+R=1$, the relevant solution is $R=u/s$. The proportion of homozygous recessives comes out to be the same as with random mating.

A more general situation in which the population may be considered to have an inbreeding and a random breeding component may be handled by using WRIGHT'S formulae (WRIGHT 1942, DOBZHANSKY and WRIGHT 1941). If F is WRIGHT'S coefficient of inbreeding and selection and mutation are operating as in the previous examples, the formulae are approximately as follows, provided s and F are small.

Genotype	Frequency	Relative selective value
AA	$p^2(1-F)+pF$	1
Aa	$2pq(1-F)$	1
aa	$q^2(1-F)+qF$	$1-s$

According to WRIGHT (1942),

$$\Delta q=pu+\frac{pq}{W}\left[\frac{(1-F)}{2}\frac{d\overline{W}_R}{dq}+F\frac{d\overline{W}_I}{dq}\right]$$

where $\overline{W}_R$ is the weighted average selective value of the random bred component, $\overline{W}_I$ is the weighted average selective value of the inbred component, and $\overline{W}$ is the weighted average of the entire population. In this case,

$$\overline{W}_R=1-sq^2 \qquad \frac{d\overline{W}_R}{dq}=-2sq$$

$$\overline{W}_I=1-sq \qquad \frac{d\overline{W}_I}{dq}=-s$$

$$\overline{W}=\text{approximately 1 (if s is small)}$$

The condition for equilibrium is that $\Delta q = O$, which leads to the equation

$$q^2(1 - F) + qF = \frac{u}{s}.$$

The left half of this equation is the expression for the frequency of homozygous recessives and the value turns out to be u/s as obtained before.

The conclusions of the preceding section still hold when there is any constant amount of inbreeding. As long as the population is at equilibrium with respect to selection and mutation pressures, the total loss in selective advantage due to the presence of homozygous recessives is $n\bar{u}$. Thus a population within which there is consanguinous mating which has survived long enough to come to an approximate equilibrium will not be at a lower level of vigor than a randomly mating population, if loss of vigor is entirely due to deleterious recessive factors. This may explain the observation that many self-pollinating plant varieties are of normal vigor.

EFFECT OF REDUCED POPULATION SIZE

Thus far it has been assumed that the population is large enough so that random fixation of alleles is not an appreciable factor in determining gene frequencies. If the population is small, random loss or fixation, which is proportional to the reciprocal of the population number (WRIGHT 1931), becomes important. Since we are here concerned with the total effect on the phenotype of many genes rather than individual effects, random fluctuations around the average value for individual loci will to a large extent cancel out. For this reason, the effect of population size in this respect is much less important than if single factors were being considered.

However, if the population is very small selection becomes ineffective and many genes become homozygous in all individuals or are lost completely. Genes having various selective values may become fixed by chance and whether a gene is retained or lost by the population may be determined more by mutation rates than by selection (WRIGHT 1931, 1937). It should be emphasized that this would be expected only in very small populations in which the population number is of the same order of magnitude as the reciprocal of the selection coefficient. In crosses between such very small populations greater increases in vigor might be obtained under the dominance hypothesis than with larger populations, though the hybrids should not be appreciably above the level of a large randomly mating population.

Some of the most striking cases of hybrid vigor, such as in hybrid corn, occur when a normally randomly mating species is inbred closely for a few generations and then crossed to another similarly inbred strain. If hybrid vigor is measured by comparing with the original open crossed strains, the conclusions previously reached still hold. On the other hand, if hybrid vigor is measured by comparison with the weakened inbred lines, the theoretical maximum increase with the dominance hypothesis may be much greater.

If a population is inbred until all individuals are homozygous for a certain

gene locus, the proportion of homozygous recessive zygotes becomes the same as the proportion of recessive genes. Thus if a normally cross-fertilizing population ($q = \sqrt{u/s}$) is inbred, without mutation or selection, the proportion of homozygous recessives approaches $\sqrt{u/s}$. The average loss of vigor in the population due to this gene is this proportion multiplied by the selection coefficient or $\sqrt{us}$.

The theoretical maximum gain in vigor in this case is the number of gene loci, n, multiplied by the average value of $\sqrt{us}$ for these loci. The average of s is not known in general, but is probably much larger than the mutation rate. The gain in vigor on hybridization therefore may be considerable. It may well be that the dominance hypothesis can account quantitatively for the observed loss of vigor with close inbreeding of normally randomly mating varieties and for its recovery on crossing, but that it can not account for any large increase beyond the level of the original outcrossed varieties from which the inbred strains were derived.

THEORIES OF HYBRID VIGOR

No discussion of such hypotheses as involve cytoplasmic factors or other non-Mendelian effects will be discussed in this paper. The entire subject of hybrid vigor has been reviewed recently by WHALEY (1944, see also GOWEN et al 1946, and RICHEY 1946).

The hypothesis that hybrid vigor is due to the dominance of favorable genes is supported by a number of observations. The frequency of recessive mutations and the correlation between recessive factors and detrimental effect have been noted in many forms. Ordinarily a cross-breeding population contains numerous gene loci heterozygous for deleterious recessive factors whose effects are concealed by their dominant alleles. Inbreeding, with its effect of increasing homozygosity, exposes some of these recessive factors and there is a resulting net loss of vigor. Crossing between such inbred lines produces hybrids in which many of the detrimental recessives are covered by dominant alleles from the other parent and an increase in vigor is the consequence. If the number of factors involved is large and there is linkage between some of them, it is improbable in the extreme that an inbred line should become homozygous only for the dominant beneficial factors and for none of the detrimental recessives. RICHEY and SPRAGUE (1931), in their experiments on "convergent improvement" of corn provided evidence that at least some of the improvement in vigor on crossing inbred lines is due to increase in the number of dominants rather than to increase in heterozygosity. Various workers on the genetics of natural populations have pointed out the large numbers of detrimental recessives that occur in nature. DOBZHANSKY, HOLZ, and SPASSKY (1942) showed that only a very small proportion (about 3 percent) of the flies in a population of *Drosophila pseudoobscura* which they studied were free of detectable deleterious recessives.

All this evidence strengthens the dominance hypothesis and it seems probable that it may explain a major part of the loss of vigor with close inbreeding of normally random mating strains and its recovery on crossing. On the other

hand, the analysis given here, if it is based on correct assumptions, shows that the dominance hypothesis cannot account for more than a small increase in vigor of hybrids whose parents are from populations which are at equilibrium. Also it cannot account for increase in vigor following the crossing of artificially inbred strains much beyond the level of the equilibrium population from which the inbred strains were derived. One might therefore look elsewhere for an explanation of causes of pronounced excess vigor under these conditions.

A second hypothesis is that heterozygosis itself produces an increase in vigor. In a sense this is a return to the original heterosis idea of EAST and SHULL which has been more recently advocated by RASMUSSON (1934) and EAST (1936). Hybrid vigor, according to this view, depends on the existence of a number of loci in which the heterozygote is superior to either homozygote and vigor then increases with the proportion of heterozygosis. Evidence for the occurrence of such loci is provided by the finding of what are apparently single gene mutations in corn which produce heterotic effects (JONES 1945). Similar findings have been made in barley by GUSTAFSSON (1946, 1947) who has emphasized the importance of factors which may produce beneficial effects as heterozygotes even when highly deleterious as homozygotes. HULL (1946) has postulated the existence of "overdominance" to explain the results of his regression analysis of corn yields and this is essentially the same idea. There are several examples of such factors in Drosophila.

This hypothesis is not subject to the quantitative limitation of the dominance hypothesis, as the following analysis of such a case shows.

Genotype	Frequency	Selective value
AA	p^2	$1-t$
Aa	$2pq$	1
aa	q^2	$1-s$

If the selection coefficients, s and t, are large in comparison with mutation rates the effect of mutation in determining gene frequencies may be neglected. The condition for equilibrium is given by

$$\frac{p}{q} = \frac{p^2(1-t)+pq}{pq+q^2(1-s)}$$

which has the solution

$$p = \frac{s}{t+s} \quad \text{and} \quad q = \frac{t}{t+s} \quad \text{(WRIGHT 1931).}$$

The average reduction in selective advantage of the population due to the two homozygous genotypes is

$$\left(\frac{s}{s+t}\right)^2 t + \left(\frac{t}{s+t}\right)^2 s = \frac{st}{s+t}.$$

Thus the loss in fitness of the population is of the order of magnitude of the selection coefficients, as has been shown by HALDANE (1937), whereas in the

previous case the loss in selective advantage due to detrimental recessives is of the order of the mutation rate. Selection coefficients generally being much greater than mutation rates, the effect of a locus at which the heterozygote is superior is much greater than that where there is a detrimental recessive. It would not require very many loci in which the heterozygote is superior to give a considerable selective advantage to a hybrid heterozygous for these loci. How many such loci exist is not known, though only a small number have been identified. However, if as many as 1 percent of the gene loci were of this type, their effect on the population would be greater than all the loci at which there is a detrimental recessive, since the ratio of mutation rate to selection coefficient must surely be of an order of magnitude less than .01.

In the case of an inferior heterozygote selection would tend to keep one or the other of the alleles in a state of almost complete fixation. This is because there is no stable equilibrium under selection alone except when one allele is completely lost (FISHER 1922, WRIGHT 1931). The population would remain in this state except for recurrent mutations which would be eliminated in the same way as detrimental dominants and the net effect on the population per locus would be of the order of the mutation rate.

Loci in which the heterozygous class is more extreme than either homozygote would require the existence of neomorphic mutations (or antimorphic, if the alleles were producing opposite detrimental effects). Such mutations have been found, though infrequently, and the heterozygous effect might be either detrimental or beneficial. However, as pointed out previously, the latter case is much more likely to be important in the population. The more distantly related two strains are, the greater is the likelihood that substitution of gene functions has occurred during the evolutionary divergence of the two. This means that in the hybrid alleles might be acting on different substrates or transforming the same substrate into different products. Such alleles would be behaving as neomorphs or antimorphs and could result in the heterozygote being more extreme than either homozygote. Another result of substitution of gene functions would be the changing of systems of gene interactions. Various kinds of complex interactions of genes from different parents might also be factors in hybrid vigor.

It is possible that increased vigor in hybrids between natural populations and recovery of vigor following the crossing of artificially inbred strains are largely due to two different phenomena. The first may be caused principally by intra- and inter-locus interactions while the second may be due to dominance of favorable genes.

SUMMARY

Assuming that all beneficial genes are completely dominant and all deleterious factors are recessive, the average decrease in selective value due to homozygous recessives is equal to the product of the number of gene loci (n) and the average mutation rate ($\bar{u}$). This is true of any population as long as it is at equilibrium regardless of the breeding structure or the amount of selective disadvantage of the individual recessive factors. Prevailing estimates of gene

number and mutation rate make it appear unlikely that the product nū is larger than .05.

If one assumes that vigor is measurable in terms of selective value, this would be the maximum possible increase in vigor under the dominance hypothesis. Hence, any hybrids between natural populations that have larger increases in vigor must be explained by other hypotheses. The likely alternative is that increased vigor is due to certain gene loci where the heterozygote is superior to either homozygote and to gene interactions. On the other hand, the dominance hypothesis may account for the recovery of the original vigor in hybrids between artificially inbred strains of normally cross-fertilizing varieties.

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THE ACTION OF ALLELIC FORMS OF THE GENE A IN MAIZE. I. STUDIES OF VARIABILITY, DOSAGE AND DOMINANCE RELATIONS. THE DIVERGENT CHARACTER OF THE SERIES[1]

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INTRODUCTION

THERE is increasing support for the notion that the gene exercises control over phenotypic expression through its mediation of specific biochemical reactions (review by BEADLE 1945). That this function is accomplished through an immediate gene-enzyme relationship is an indirect inference from the recent abundant evidence from work on Neurospora in which, in the presence of an immediate precursor, the success or failure in producing a specific chemical compound has been correlated with alternative forms of a single gene (HOROWITZ, BONNER, MITCHELL, TATUM and BEADLE 1945; SRB and HOROWITZ 1944). There is direct evidence for this view from several studies which establish the dependency of the presence of a particular enzyme on the dominant form of a single gene (see GARROD 1923; SAWIN and GLICK 1943; ATWOOD and SULLIVAN 1943).

For the student of biosynthesis the genetic technique is a valuable tool since it allows ultimately the step-wise interruption of reaction chains. To establish the details of any one of a chain of reactions requires only a single alternative form of the wild type gene. Whether this mutant is, in the terminology of MULLER (1932), an amorph (showing complete absence of type effect) or a hypomorph (having reduced type effect) is of secondary concern. Because of its ease of identification the amorph may even be preferred. Indeed, the methods employed in identifying the induced mutants in the Neurospora experiments (BEADLE and TATUM 1941) suggest that if not deficiencies, these mutants are almost certainly amorphs.

The student of physiological genetics, however, who may be concerned more immediately with particular relations between gene and gene-product than with reaction sequence, is obliged to consider the likelihood that a gene in control of a specific biosynthetic reaction in Neurospora, like many other genes (BRIDGES and BREHME 1944; EMERSON, BEADLE and FRASER 1935) may have a variety of mutant forms. If such mutant forms exist the question arises whether they differ in effect from one another only in the degree of divergence from wild-type effect which they produce. In several cases in which more than two alternative gene forms have been studied intensively their action

[1] This investigation was conducted at the UNIVERSITY OF MISSOURI, Columbia, Missouri.

is not interpretable in terms of so simple a scheme (review by WRIGHT 1941, 1945; Stern 1943). In some of these cases, effects other than type have been described for certain alleles, a characteristic which has led MULLER (1932) to call them neomorphs. In other cases, in which all of the alleles show varying degrees of the same type effect their behavior in combinations and in dosage studies contradicts the notion that the alleles differ only to the extent that they affect the strength of a single primary reaction. Attempts to view such results in terms of direct gene-enzyme relations have forced the consideration that alleles may differ individually in their relative effects on both phases of some type of two-dimensional scheme of reaction. On this basis qualitative differences between enzymes traceable to differences between alleles would be held to influence independently the rate at which union with substrate is accomplished and that at which the resulting complex is degraded to yield the immediate product. If it is assumed that alleles may differ in chemical configuration they may determine different immediate products through interaction with a single cellular substrate. They may even be specific for related but different substrates.

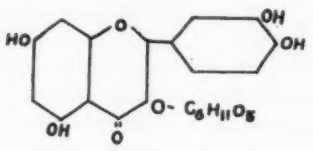
These concepts have developed largely from genetic experiments, and thus far their confirmation has not been sought in experiments designed to test them directly. On the other hand, those studies which afford a clearer view of genic action as it affects specific reactions involving known substrates and products are without the benefit of evidence on more than two alternative gene forms of a single locus.

Such studies, because of their limited scope, may underestimate the complexity of genic action. Genetical and biochemical investigation of the action of genes with a variety of mutant forms, on the other hand, may be expected to narrow the limits within which a hypothetical picture of genic action can be framed.

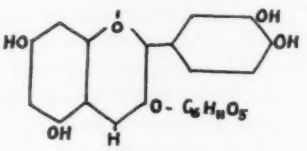
For such a combined attack the *A* alleles of maize provide especially favorable material. In suitable genetic background the various forms of this gene determine correspondingly varying amounts of purple pigment (anthocyanin) in certain tissues and of purple and brown pigments in others. These pigments are produced abundantly, in most organs to the extent that quantitative and qualitative studies are feasible on an individual-plant basis. Moreover, there is some basis for a view as to the specific action of *A;* SANDO et al. (1922, 1932) isolated from the brown plant (*a a B Pl*) a flavonol identified as isoquercitrin (I) and from the purple plant (*A A B Pl*) an anthocyanin identified as chrysanthemin (II). The former differs from the latter only in the substitution of an atom of oxygen for one of hydrogen in the same position, and it would seem from this that the *A* gene is concerned with the specific hydrogenation of some unknown precursor which isoquercitrin and chrysanthemin have in common. While more recent evidence, the subject of a succeeding paper in this series, would allow this as only one of a number of equally plausible interpretations, the advantage of working with identifiable chemical substances which are more or less immediate products of genic action and which are produced

in quantities sufficient for direct study strongly recommends this locus for investigation.

The production of brown pigment which is associated with various mutants of *A* and present to the exclusion of purple pigment in *aa* plants is entirely passive as regards the *A* gene. For, as will be reported in detail below, maize tissue which carries a homozygous deficiency of the *A* locus produces typical brown coloration. It appears, therefore, that the amount of brown pigment



I



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provides a measure of the extent to which a specific substrate remains untransformed by the action of the *A* gene or gene product. The occurrence and easy identification of a substance which is alternative to that associated with wild type action is a feature displayed by few genes whose mutant forms have been described. Several hypotheses, advanced to account for the anomalous behavior of multiple alleles, (WRIGHT 1941, 1945; STERN 1943) share the assumption that alleles may differ in their efficiency of substrate conversion as well as in that of combination with substrate. In the case of the *A* alleles, the occurrence of the alternative product affords an opportunity to distinguish between the two possibilities.

The variety of multiple alleles together with the potentialities for the production of new alleles via spontaneous and induced mutation at the *A* locus further suit it for studies on genic action. The mutation of *a* under the influence of the *Dt* gene (RHOADES 1941) yields mutants which have in several cases been shown to be distinguishable in their action. Likewise, the mutants obtainable in relatively high frequency from A^b by spontaneous mutation (STADLER 1941) appear to constitute a heterogeneous series. Any hypothesis which seeks to explain *A* action must thus meet the rigorous test imposed by evidence on an assortment of alleles.

The study of this series of alleles has interesting implications as to the course of biosynthesis in as much as a number of other genes whose mutant forms interrupt or modify the production of anthocyanin in maize have been identified. Each of these genes presumably is in control of one of a number of chemical reactions leading ultimately to the formation of the purple pigment. Moreover because of the relative ease of identifying mutants in maize, the isolation of other genes which control anthocyanin pigmentation should not be difficult.

The present paper reports the results of a genetic investigation of variability, dosage and dominance relations of a number of *A* alleles. In the opinion of

the author, the data are not in agreement with expectations on the basis of any simple unilateral action of the gene. The action of at least one group of *A* alleles, which share a common geographical background, does not support the notion of a one to one correspondence between the gene and the biochemical reaction. Succeeding papers in this series will report chemical investigations of the pigments affected by the action of the *A* alleles.

HISTORICAL

The mutant gene *a* was described by EMERSON (1916 unpublished, 1918, 1921) as associated with the production of brown pigment in certain vegetative organs of the maize plant, notably husks, sheaths, and glumes, in the presence of the complementary color factors *B* and *Pl;* brown pericarp in the presence of the complementary factor *P*; and colorless aleurone in the presence of the then known complementary factors for color in that tissue, *R*, *C*, and *ii*. The type allele, *A*, with the same background of complementary factors, produces purple plant parts, red pericarp color, and purple or red aleurone color depending on whether the plant carries *Pr* or *pr*, respectively. Where differences in pigmentation were shown to be due to *A* versus *a* constitution, the former showed dominance over the latter.

Two alleles from South America have been described and demonstrated to be allelic to *a* (EMERSON and ANDERSON 1932.) A^b, collected from Ecuador, was described as indistinguishable from the original *A* except in its determination of brown pericarp pigment. This effect is dominant to the red of *A* and completely linked with the A^b allele. The gene a^p, collected from Peru, produces pale-purple aleurone color, a brown pericarp color indistinguishable from that of A^b, and a reddish-brown coloration of vegetative parts. The action of a^p resulting in intermediate coloration of the aleurone and plant was reported as dominant to that of *a*, but recessive to that of *A* and A^b. However, in its effect on pericarp color a^p is completely dominant to *A*.

More recently, RHOADES (1941) has reported the reversion of *a*, through *Dt* action, to five phenotypically distinct alleles. The most frequently recurrent of these resembles *a* with the exception that it no longer shows typical response to *Dt*. A second and recurrent type of reversion produces an allele which is indistinguishable from the originally described *A*. Two other alleles designated A^{br} and A^{rb} differ from *A* only in their respective determinations of brown and red-brown pericarp color with *P;* in both cases the pericarp color effect is recessive to the red color effect of *A*. Still another allele arising from *Dt* action on *a* resembles a^p in plant and aleurone color but produces a brown pericarp color which is recessive to the red effect of *A*.

Additional alleles have been obtained and described by STADLER (1941, 1943), who showed that A^b mutates spontaneously at a rate approximating 1/1000 to types resembling a^p in plant phenotype. Genetic tests of nine such mutants showed that all retain the brown pericarp effect of the parent A^b allele and, as in the case of A^b, all are dominant to *A* in this respect. There are apparent differences between some of the newly described mutants in the

depth of pigmentation in plant and aleurone parts which they determine, but all have a stronger effect in this respect than the original a^p. A^w-U1, an "intermediate" allele, was obtained from A by ultraviolet treatment of pollen. The same experiment yielded three mutants which are completely haploviable and indistinguishable from a except in their complete lack of response to the *Dt* gene. X-ray treatments of pollen carrying A have yielded three mutants resembling a phenotypically, but strongly indicated as deficiencies on the basis of work by STADLER and ROMAN (1948), showing a lowered gametophytic transmission and associated chlorophyll and viability effects for these mutants. Finally, STADLER has recovered from a, by *Dt* action, an "intermediate" mutant designated A^w-D1, which apparently is quite similar to the intermediate which RHOADES describes.

The behavior of some of these newly acquired alleles in compounds (STADLER 1943) is of interest here. In hybrids with a the mutant derivatives of A^b (designated A^d) are completely dominant and in F_2 the phenotype of A^d/a is indistinguishable from that of A^d/A^d. The same is true of a^p. Whereas the mutants A^w-D1 and A^w-U1 produce a redder plant than a^p, the hybrids with a^p are intermediate. No such effect of the a^p allele was indicated in its combinations with A or A^b, but with A^{br}, distinguished at the time from the former alleles only by its recessive brown pericarp, the a^p gene produces an intermediate phenotype. In combinations with A^{br} an intermediate effect is produced by each of the four mutant pales from A^b; but the combination of A^{br} with A^w-U1, the "pale" derivative from A, was described as indistinguishable from A^{br}/a sib plants.

MATERIALS AND METHODS

The alleles of A which were employed throughout the genetic investigations are listed in table 1. As a result of the genetic studies several of the alleles listed in table 1 as associated with purple plant phenotype have been shown to be weaker in effect (associated with reduced anthocyanin content) than A-st. Likewise, the alleles whose effects place them in the intermediate class do not comprise a homogeneous lot. Hence, the separation of the A alleles into the three classes is an arbitrary one; nor is the order in which these alleles occur within each phenotypic class of table 1 to be interpreted, necessarily, as the order for their phenotypic effects.

Considerable variation in pigmentation may occur due to modifier action. Throughout this investigation, comparisons have been limited to those between plants within the progenies of a single cross.

The X-ray mutant, *et*, located on the third chromosome approximately twelve crossover units from a was employed as a marker in the crosses reported here. Seeds homozygous for this factor have an etched endosperm and produce virescent seedlings. While the endosperm phenotype is somewhat variable and in some backgrounds may not be totally classifiable, the virescent character permits fully reliable separations.

Since the crossing techniques employed in the genetic studies differ for the

several phases of the investigation, these are described separately in the chapters dealing with experimental results.

TABLE 1

Sources and phenotypic effects of the A alleles under investigation.

ALLELE	PHENOTYPE		SOURCE
	PLANT	ALEURONE	
A	purple	purple	Common type in N. America
A-st	"	"	Standard extraction from *A*
A^b	"	"	Collected from Ecuador
A-D1	"	"	By mutation from *a* through action of *Dt* Gene
A-D2	"	"	"
A-D3	"	"	"
A-D6	"	"	"
A-D7	"	"	"
A^{rb}	"	"	"
A^{br}	"	"	"
A^w-D1	intermediate	intermediate	"
A^w-U1	"	"	Ultra-violet induced mutant from *A*
A^d-31	"	"	From A^b by spontaneous mutation
A^d-41	"	"	"
a^p	"	"	Collected from Peru
a	brown	colorless	Nebraska (single plant)
a-X1	brown in viable sectors (zygotic lethal)	"	X-ray deficiency from *A*

THE ACTION OF *A*

The *A* gene is one of a number of factors at different loci which are concerned with the development of anthocyanin. The action of alleles of any one of these factors, therefore, must be studied under conditions in which the background of complementary factors is well defined and constant. In the present study, all of the complementary genes necessary for anthocyanin development in endosperm and plant tissue were maintained homozygous. The genes *b* and *pl* frequently were segregating but the data involving plant pigmentation are drawn exclusively from *B Pl* individuals. The three phenotypic classes which are associated with *A*-factor differences and to which reference has already been made in table 1 are:

Purple. Plants in this class show deep purple pigmentation of sheaths, husks, brace-roots, culm, glumes, and anthers. Aleurone tissue is likewise deep purple and pericarp color in the presence of *P* may be either red or brown.

Brown. Anthocyanin pigment is absent in plants of this class. Sheaths, husks, brace-roots, glumes, and pericarp tissues are pigmented brown; aleurone cells are devoid of pigment.

Intermediate. This class includes those plants which show intermediacy between the two classes above. Vegetative organs are reddish-purple or reddish-brown, suggesting a mixture of the purple and brown pigments. The cells of

the aleurone may range in color from dilute-purple to very pale and brown pericarp color is the rule.

Whatever the specific biosynthetic function of the *A* alleles may be, it is clear that they are concerned ultimately with the determination of varying amounts of purple pigment in the cells of certain tissues. It is not correspondingly clear, from visual observation, that the occurrence of brown pigment is affected; the purple pigment is produced in such concentration in plants of the purple class as to mask any brown pigment which may be present.

Histological studies carried out with sheath tissues of representative color types have given a clearer picture of *A* action. Sections of sheath tissue taken from purple plants carrying the *A*-st allele show deep purple pigmentation of the cells immediately surrounding the bundles and of the epidermal, hypodermal, and mesophyll cells. The first traces of anthocyanin develop in the thick-walled cells surrounding the bundles and the order of its first appearance in the other tissues is that given above. In sheath material of brown plants having the genetic constitution *aa*, the distribution of brown pigment among the various tissues is a close parallel to that of anthocyanin in the purple type; likewise, the order of development of the brown pigment in *aa* sheath tissues corresponds closely to that of the purple pigment in *A*-st. Anthocyanin is always found within the vacuole of the cells in which it occurs whereas the brown pigment appears to be localized completely in the cell walls and the cytoplasm.

The occurrence of the purple and brown pigments in mutually exclusive regions of the cell facilitates the study of the *A* alleles from the standpoint of their effect on both pigments. Brown sheath tissues from *aa* plants are without the faintest trace of anthocyanin, a result which was anticipated from their macroscopic appearance. Purple sheath tissues from *A*-st plants show a total absence of brown pigment in cell walls and cytoplasm, the latter appearing colorless under the microscope. The sheath cells of plants carrying an allele of *A* which determines intermediate plant color have a combination of brown wall pigment and dilute purple or red vacuolar pigment.

The differences in relative concentration of the wall and vacuolar pigments associated with different, intermediate *A* alleles are paralleled by differences in the relative times of their first appearance. The vacuolar pigment of intermediate types is not only less intense than that of the purple types but tends to appear later in the history of the individual sheath; conversely, the brown pigment of *aa* plants appears earlier than that of the intermediate types.

These observations lead to the conclusion that the *A* alleles determine not only varying levels of purple pigmentation but of brown pigmentation as well. In general, an allele which is potent in its capacity for anthocyanin production is associated with the development of little or no brown pigment, and *vice versa*. This need not imply necessarily that all, or any one, of the *A* alleles actively determines the production of both purple and brown pigments. For, since the concentrations of purple and brown pigments associated with the

various alleles of *A* appear to be negatively correlated, it is reasonable to inquire whether the production of brown pigment may not be passive as regards the action of these alleles. In this regard it should be noted that typical brown pigmentation may occur in cells which are deficient for the *A* region of the chromosome. This evidence comes from plants carrying a deficiency for the *A* locus on both third chromosomes but having the deficient region covered by an unstable duplication carrying the A^b gene and having a centromere. In these plants sectors of anthocyanin-free tissue frequently occur due to the occasional loss of the A^b-bearing duplication. Microscopic studies of these deficiency sectors have shown the development of brown pigment there to be normal and characteristic of that in *aa* plants.

A tentative hypothesis of *A* action is presented at this point, since it provides a basis for evaluating the genetic evidence which follows. According to this hypothesis, in the absence of an *A* gene, a series of reactions in the maize cell leads to the formation of brown pigment. The *A* alleles bring about the conversion of one of the precursors of brown pigment to a succeeding precursor of purple pigment, and the strength of this reaction varies for the different alleles of the series. In the case of *A*-st, the conversion is strong enough to preclude the formation of even a trace of brown pigment. The reaction in the case of alleles associated with intermediate effects is weaker, with the result that both brown and purple pigments are produced. In *aa* cells the conversion reaction producing anthocyanin precursor may be presumed either not to occur or to proceed at a rate below a certain critical threshold, thus accounting for the production exclusively of brown pigment by these cells.

THE VARIABILITY OF THE *A* ALLELES

All of the *A* alleles in table 1 were tested for differences by comparing them in backgrounds designed to reduce the variation due to modifiers. Two types of crosses were employed for this purpose. The one most often used was the backcross to *et a*/*et a* of the marked compound carrying the comparison alleles; the latter may be designated A^1 and A^2 for illustrative purposes in the following cross:

$$et\ A^1/Et\ A^2 \times et\ a/et\ a \qquad \text{(Type Cross I)}.$$

Some use was made of the cross between two heterozygotes:

$$et\ A^1/et\ a \times Et\ A^2/et\ a \qquad \text{(Type Cross II)}.$$

Seeds produced from either of these crosses and having the constitutions *et* A^1/*et a* and *Et* A^2/*et a* are separable on the basis of their etched (*et*) or normal (*Et*) appearance and may be planted in separated progenies for comparison studies.

Crossing over between *et* and *a* (approximately 12 percent) results in some *Et* A^1/*et a* and *et* A^2/*et a* plants. Progenies planted from *et* seeds therefore contain mainly *et* A^1/*et a* plants along with a small proportion of *et* A^2/ *et a* individuals; similarly progenies planted from *Et* seeds have a small proportion of *Et* A^1/*et a* individuals among larger numbers of *Et* A^2/*et a* plants. The recogni-

tion, on the basis of plant phenotype, of these crossover individuals, whose genotypic constitution could be verified later, has in many cases confirmed differences between two *A* alleles which were noted from a comparison between the two progenies.

A modification, illustrated below, of Type Cross I

$$et\ A^1/Et\ A^2 \times et\ a^p/et\ a^p$$

has been successful in establishing differences between alleles which determine purple plant color but are not readily separable in progenies from Type Crosses I or II. Individuals having the genetic constitutions *et* A^1/*et* a^p and *Et* A^2/*et* a^p are separable, as before, on the basis of their etched and normal phenotypes, respectively. The increased utility of this cross for comparison purposes is a result of the tendency shown by a^p to reduce the anthocyanin-producing capacity of the A^1/a^p, as compared with the A^1/a or A^1/a-X1 individual. This phenomenon, which has been called "competition," is discussed more fully in the later chapter dealing with compounds.

The Alleles of the Purple Class

The effects of A^b, A^{br}, and A^{rb} on pericarp color were compared with that of *A*-st in sib progenies. A^b and A^{br} are distinguishable, on the basis of their brown pericarp effect, from *A*-st which is associated with red pericarp color. A^{rb} is likewise separable from *A*-st by its red-brown pericarp effect. A^b and A^{br}, both of which have had wide crossing, have never failed in their determination of brown pericarp, neither when homozygous nor in hybrids with *a*. Likewise *A*-st has been consistent in its red-pericarp effect. These results are in agreement with the early description of A^b (EMERSON and ANDERSON 1932) and of A^{br} and A^{rb} (RHOADES 1941).

The alleles A^b and A^{br} are distinguishable from *A*-st in their effects on plant color also. In comparisons between A^{br}/a and *A*-st/*a* plants, the former consistently show slower and weaker development of anthocyanin than their *A*-st/*a* sibs; this difference is first manifested in the coleoptile and sheaths of the seedlings and is evident at maturity in the sheath and husk tissues. Classification of these differences is most reliable at flowering, however, when the sheaths of *A*-st/*a* plants are deep purple in color as compared with those of A^{br}/a which are lighter and more reddish-purple in appearance. The increased pigmentation which occurs as these plants approach maturity reduces, rather than enhances, the difference between the A^{br}/a and *A*-st/*a* individuals. A^b/a plants are likewise distinguishable from sib *A*-st/*a* plants at flowering by the more reddish-purple appearance of the sheaths.

In view of the fact that these color differences are visible to the eye only in certain of the pigmented regions of the plant and then only when the plants are under close scrutiny, more conclusive evidence of differences between these alleles was sought. Two types of approach have been particularly useful for this purpose and promise to be valuable in further work: (1) objective classification and (2) chemical spot-testing.

The objective technique has as its central feature the classification, on the basis of suspected differences, of individual plants in a population, the *A* constitution of the individuals remaining unknown to the operator at the time of classification. Every opportunity is afforded the operator to perceive and study differences in known progenies before the classification of unknowns is attempted; in practice, the study of known progenies has not only cultivated a familiarity with phenotypic differences in the progeny, but, since pigmentation differences vary with age of the plants, has frequently suggested the most desirable growth stage for successful classification.

The method may be illustrated by considering its application to the comparison of sib *A*-st/*a* and A^{br}/a plants. These individuals came from the cross:

$$et\ A\text{-st}/Et\ A^{br} \times et\ a/et\ a.$$

TABLE 2

The results of the objective classification of A-st/a and A^{br}/a plants occurring as unknowns in the progeny from the cross: et A-st/Et A^{br} × et a/et a

PROGENY TOTAL	CLASSED *A*-st/*a*			CLASSED A^{br}/a		
	CORRECTLY	IN-CORRECTLY	P	CORRECTLY	IN-CORRECTLY	P
42	17	3	<0.01	20	2	<0.01

Etched seeds (*A*-st/*a*, except for crossovers) and normal seeds (A^{br}/a, except for crossovers) were planted in separated progenies for observation. In addition, a randomly chosen sample of seeds from the same ear was planted. Observation of the known progenies suggested that classifiable differences were present at flowering time in the sheath color (as before, the basis for this difference was a purple versus a reddish-purple color in this organ) and accordingly, classification of the unknown progeny for sheath color differences was attempted at this stage. The results of the classification are presented in table 2, expressed in terms of the number of individuals placed correctly and incorrectly in each genotypic class as determined by pericarp color at maturity. Values of P were determined by the Chi-Square test, the hypothesis adopted being one of absence of classifiable differences in the unknown progeny. The null hypothesis is untenable and a real difference between *A*-st/*a* and A^{br}/a in plant color effect may thus be considered demonstrated.

The chemical spot-testing technique has been successful in identifying differences between *A*-st and A^{b} and in confirming the differences in effect between *A*-st and A^{br}. This test depends on the presence in purple husk tissue of two types of substances with regard to coloration produced under basic conditions; one of these gives a yellow color, the other, anthocyanin, gives a blue color when treated with dilute potassium carbonate solution. Under the

same conditions, a mixture of the two pigments gives a green color, the shade depending on the ratio of the concentrations of the two types of pigment. Since the *A* alleles are concerned ultimately with changes in this ratio, the spot test furnishes a convenient method of measuring differences between them. In actual practice, husks from plants of known genotype are collected, recorded, and treated as unknowns in the testing. Under standardized procedure, the husks are extracted with water and the filtered solution acidified slightly with hydrochloric acid. Strips of filter paper are dipped into the respective solutions and are classified for yellow versus bluish shade of green produced on the addition of a drop of potassium carbonate solution. Sharp, consistent differences in shade which coincide with differences in *A* locus constitution may be taken as evidence for the individuality of the alleles under consideration.

The spot test method was applied to the measurement of differences between *A*-st and A^{br}; husk tissue from ten sib plants, five of which were *A*-st/*a* and five, A^{br}/a, was used. The shades of green produced by the two types were easily separable and spot tests of the ten individual samples were repeatedly classified without error. The same technique was applied under entirely analogous conditions to husk tissue of sib *A*-st/*a* and A^{b}/a individuals and showed differences as distinctive as those of the *A*-st versus A^{br} comparison. The spot tests of both A^{br}/a and A^{b}/a individuals were characteristically yellow-green in color as compared with the blue-green spot tests of extracts of sib *A*-st/*a* plants.

The individuality of *A*-st and A^{br} in their effects on plant pigmentation has been fully supported by comparisons involving the combination of these alleles with a^{p} instead of with *a*. Individuals having the genetic constitutions *A*-st/a^{p} were separated from A^{br}/a^{p} individuals on ears produced from the cross:

$$et\ A\text{-st}/Et\ A^{br} \times et\ a^{p}/et\ a^{p}.$$

It was apparent from a study of the known progenies that the combinations with the a^{p} allele provide a highly sensitive background for detection of differences between *A*-st and A^{br}. At the flowering stage the sheaths, husks, glumes, and anthers of A^{br}/a^{p} plants are maroon in color; sib *A*-st/a^{p} plants on the other hand are deep-purple in these regions. The differences between these types remain distinct throughout later growth stages and are evident at maturity.

Differences between *A*-st and A^{rb} in plant color effect have not been identifiable in hybrids with recessive *a*. In combinations with a^{p}, however, differences of the same type and order as those described above for the comparison between *A*-st and A^{br} in this background are evident.

Each of the "purple" alleles *A*-D1, *A*-D2, *A*-D3 and *A*-D6 was compared with *A*-st in hybrids with *a*. While these alleles share with A^{br} and A^{rb} an origin from *a* by independent mutation induced by *Dt* action, they consistently are associated with red pericarp color; in this regard they differ from A^{br} and

A^{rb} and are indistinguishable from A-st. No differences were noted between these alleles and A-st in their effects on pigmentation of vegetative organs.

The Alleles of the Intermediate Class

The alleles a^p, A^d-41, A^d-31, A^w-D1 and A^w-U1 are associated with intermediate pigmentation (table 1). Each was compared with A-st in sib progenies originating from either Type Cross I or II or both. In these marked progenies A-st/a plants consistently developed red pericarp color and the full purple plant and aleurone phenotype. Without exception, plants carrying "intermediate" alleles produced brown pericarp color and sheath, husk, glume, anther, and aleurone regions showed much weakened anthocyanin content as compared with A-st/a sib plants.

An analogous series of comparisons originating from crosses between the five "intermediate" alleles and A^{br} were studied for differences; the results indicate that A^{br} has only its brown pericarp effect in common with the alleles of the intermediate class. The latter are as distinct from A^{br} in their effects on plant and aleurone pigmentation as they are from A-st.

Within the intermediate class of alleles several different levels of action are represented. Plants having the constitution a^p/a are almost brown in appearance, the sheaths, husks and glumes showing only faint anthocyanin coloration; the anthers are devoid of color and the aleurone cells are pale purple. In some backgrounds, a^p/a plants have appeared so weak in anthocyanin production as to be inseparable from a/a plants except in very late stages of growth. In contrast, A^d-31/a plants are pigmented reddish-purple in their vegetative regions, including the anthers, and develop a much deeper aleurone color than sib a^p/a plants. Measured in the same background, the effect of A^d-41 on both plant and aleurone color is intermediate between that of a^p and A^d-31.

Microscopic studies of the sheath tissues of a^p/a, A^d-41/a and A^d-31/a plants have shown these types to differ in the relative amounts of red and brown pigments in individual cells. Moreover, the times at which the red and brown pigments make their first appearance, relative to one another, are characteristic for each; thus, in a^p/a plants, brown wall pigment develops in the early stages of the growth of the sheath, while the first appearance of anthocyanin is several weeks delayed over that of the brown pigment; at maturity, anthocyanin pigment is restricted to the epidermal cells and to the single layer of cells immediately surrounding the bundles where its concentration is low. In A^d-41/a plants, the first development of anthocyanin in sheath tissues is delayed a week or ten days beyond the time of first appearance of brown pigment; its concentration in the epidermal and bundle sheath cells at maturity is greater than in the case of a^p/a sheath material, and in addition some anthocyanin develops in the hypodermis and more rarely in the mesophyll cells. In the case of A^d-31/a plants, the first development of both anthocyanin and the brown pigment starts at approximately the same stage in the development of the individual sheaths and at maturity the pigmented tissues have relatively more anthocyanin than either a^p/a or A^d-41/a plants.

Each of the alleles, A^w-D1 and A^w-U1, was compared with a^p, A^d-41, and A^d-31 in sib progenies originating from back-crosses of the respective, marked compounds to *et a*/*et a* individuals (Type Cross I). The development of brown pericarp color was associated with all of these alleles. A^w-D1/*a* and A^w-U1/*a* individuals show relatively strong anthocyanin coloration of plant and aleurone parts and in this respect are clearly distinguishable from a^p/a and A^d-41/*a* plants. Comparisons of A^d-31/*a* with A^w-D1/*a* and A^w-U1/*a* individuals, suggest that these alleles are similar and inseparable in their pigmentation effects; the evidence on dosage and combinations of alleles which follows indicates, however, that A^d-31, like a^p and A^d-41, is distinguishable from A^w-D1 and A^w-U1.

THE EFFECTS OF DIFFERENT DOSES OF INDIVIDUAL *A* ALLELES

Evidence on gene dosage was obtained largely from comparisons of A^1/A^1 and A^1/a-X1 individuals, where, for purposes of illustration, A^1 may be considered to represent the allele whose dosage effects are under investigation. *a*-X1 is an X-ray induced, homozygous-lethal deficiency showing normal transmission through female germ cells and slightly lowered transmission through the pollen (STADLER and ROMAN 1948). The comparison types represented above are obtained as sib individuals by planting separated progenies of etched (*et*) and normal (*Et*) seeds from selfed ears of plants having the genetic constitution *et* A^1/Et *a*-X1. Except for crossovers, virescent plants (from etched seeds) are A^1/A^1, while normal seedlings (from normal seeds) are A^1/a-X1 in constitution, and the separated progeny may be studied for the effect of one versus that of two doses of the particular *A* allele involved. Because of the triploid nature of the endosperm tissue, the effect on aleurone color of three dosage levels may be studied.

Comparisons between A^w-D1/A^w-D1 and A^w-D1/*a*-X1 individuals were made in several progenies. A cumulative action of A^w-D1 in its effect on the pigmentation of plant and aleurone was evident in each case; plants carrying two doses of this allele produce greater amounts of anthocyanin than those with one dose. In the aleurone, three doses of A^w-D1 consistently produce a deeper colored tissue than either one or two doses. In vegetative tissues, these differences are visible as early as the five- or six-leaf stage when the anthocyanin pigmentation in sheaths of homozygous individuals is stronger and shows a fuller coverage of the organ than that of the hemizygotes. Observation of the plants at flowering reveals corresponding differences in the husks, glumes and anthers of the two types. At maturity A^w-D1/A^w-D1 plants are pigmented a reddish-purple while A^w-D1/*a*-X1 plants have less purple color, appearing brownish-red in these regions. The individual cells in sheath sections taken at flowering from plants carrying two doses of A^w-D1 were observed to be of a distinctly deeper shade of red and appeared lighter brown in the cell-wall regions than those of sib, hemizygous plants.

A cumulative effect on plant and aleurone pigmentation has been established also for A^w-U1 in progenies where sib plants carrying one versus two doses of this allele could be compared. The differences are of the type and

magnitude reported above for A^w-D1 which is not surprising since these two alleles, in combination with *a*, proved inseparable in their effects.

The action of the "pale" alleles, a^p, A^d-41 and A^d-31 is in direct contrast to that of A^w-D1 and A^w-U1. Progenies testing the dosage effect on plant pigmentation have failed repeatedly to show any cumulative action for these alleles. With respect to the pigmentation produced in vegetative tissues, a^p/a^p plants are indistinguishable from a^p/a-X1 individuals. In aleurone tissues where the effects of one, two and three doses of a^p gene were compared, there was no change of color phenotype with increasing doses. The alleles A^d-41 and A^d-31, which differ from each other and from a^p in their determination of distinct levels of pigmentation, also have no cumulative effect with increasing doses.

The failure to show such an effect is not due to lack of opportunity for the observation of such effects. Seeds on selfed ears of the hemizygotic plants frequently show differences in depth of pigmentation though never of a degree approaching those which are determined by different doses of A^w-D1 or A^w-U1. In the cases in which classification for such differences has been made, progeny tests not only failed to show a correlation between level of pigmentation of vegetative parts and that in the aleurone but indicate that the differences in intensity of pigmentation in the aleurone tissue are without relation to the level of dosage of the "pale" alleles in question.

The "purple" alleles, *A*-st, A^b, *A*-D1, and *A*-D2 were investigated for cumulative effects on both plant and aleurone pigmentation. In all cases, plants carrying a single dose of these alleles in combination with the deficiency, *a*-X1, are full-purple in phenotype and indistinguishable from sib plants carrying two doses of the respective alleles. Similarly, in the aleurone where three dosage levels were compared, no differences were observed. In these cases the failure to show dosage effects cannot be considered convincing evidence for their absence. A single dose of these alleles produces intense purple coloration of aleurone and plant tissues and it would not be unreasonable to argue that this concentration of pigment is sufficient to mask, to the eye, any increased effect due to additional doses of the allele. The answer to this problem awaits the application of more direct methods for measurement of the pigments involved.

The use of a deficiency instead of the recessive form of the gene in dosage studies is preferable on the grounds that it escapes any interpretations which might be made on the assumption of a positive action of the recessive allele. In this connection evidence of exaggeration effects of the *a*-X1 deficiency is entirely lacking in spite of repeated opportunities for its observance:

(1) a/a plants are indistinguishable from a/a-X1 individuals in all regions of the plant which are sites of *A* expression.

(2) In the preparation of genetic material for dosage experiments various plants carrying a number of alleles, here designated A^*, have been crossed with a/a-X1 individuals to obtain the A^*/a-X1 types for selfing. The selfing plots contained both A^*/a and A^*/a-X1 plants which should have afforded the opportunity to observe a positive effect of the *a* gene if present. None was

observed though there were included ample numbers of "intermediate" *A* alleles, which should provide the most sensitive background for the observation of such an effect.

(3) Reference has already been made to the evidence from plants carrying a ring which covers the doubly-deficient *A* locus. The occurrence in these plants of brown pigment in occasional sectors, the cells of which are deficient for the *A* locus, is characteristic of that which occurs in *aa* plants.

Considered from another standpoint the studies involving *a* rather than *a*-X1 are a desirable supplement to the deficiency studies. The deficiency region which involves *a*-X1 is known to include at least two other factors (STADLER and ROMAN 1948) and may involve still other unidentified genes. In the studies involving the deficiency, therefore, the *A* gene under consideration is not the only one which is accumulated; two, or possibly more, neighboring genes are also involved. Hence, differences which are correlated with doses of the deficiency segment may never be attributed, unreservedly, to dosage of the test gene itself. The evidence from "dosage" studies involving *a* instead of *a*-X1 disallows any interpretations on the basis of dosage effects of alleles other than the test gene.

These results showing *a* to be indistinguishable from the deficiency *a*-X1 and therefore without any degree of type effect, argue strongly for the interpretation of the results of the dominance studies with recessive *a* as true effects of dosage of the dominant genes concerned.

All of the alleles which were tested in the dosage experiments using *a*-X1 were tested in an analogous series of comparisons involving the use of *a*. The results were comparable in all respects. The alleles, A^{w}-D1 and A^{w}-U1, which showed cumulative action in the dosage experiments, showed incomplete dominance over *a* in plant and aleurone tissues. On the other hand, a^{p}, A^{d}-41 and A^{d}-31, all of which were previously shown to be without dosage effect, showed complete dominance over *a*.

Additional alleles, for which dosage evidence involving the use of a deficiency is lacking, were tested for their dominance relations with *a*. These include three alleles, A^{d}-22, A^{d}-222, and A^{d}-212, all of which originated from A^{b} by spontaneous mutation, and A^{br}, previously described. The first three of these showed no differences between homozygote and heterozygote in plant and aleurone color effects. In the cases of A^{d}-22 and A^{d}-212 there was a segregation for color level among seeds on selfed ears of plants heterozygous for *a;* as before, however, progeny tests proved that these differences bore no relation to dosage levels of the test genes. A^{br}, however, was shown to lack complete dominance over *a*.

With regard to the six pale alleles which have no dosage effects several points merit further emphasis:

(1) These alleles are all of South American extraction. a^{p} was found in material accessioned from Peru; the remaining five are mutants from A^{b} which came originally from Ecuador.

(2) It is impossible to explain the negative results reported for these alleles

in terms of masking effects of the type discussed above in connection with the alleles of the purple class. The a^p alleles in question are associated with intermediate levels of pigmentation which should provide a most sensitive background for the recognition of differences. Moreover, as reported in the preceding section, three of these alleles have been shown to be distinct in their determination of pigmentation levels. A^d-31, the most potent of these, is indistinguishable from A^w-D1 and A^w-U1 in color effect. Yet, it will be remembered individuals carrying two doses of A^w-D1 or A^w-U1 in vegetative tissue and two and three doses of these alleles in the aleurone cells are readily separable from those carrying one dose.

(3) The behavior of these alleles poses a contradiction to the simple hypothesis proposed in a preceding chapter to account for the action, on a biochemical level, of the *A* alleles. Discussion of this point will be considered in detail in a later section.

THE EFFECTS OF VARIOUS COMBINATIONS OF ALLELES

The behavior of the *A* alleles in various combinations was studied in progenies which, in addition to the compound represented here, for purposes of illustration, as A^1/A^2, provided the corresponding heterozygotes A^1/a and A^2/a for comparison. Plants having these respective genotypes were obtained from the following cross:

$$et\ A^1/et\ a \times Et\ A^2/et\ a. \qquad \text{(Type Cross II)}$$

Except for crossovers, etched (*et*), colored seeds from this cross produce plants having the genetic constitution A^1/a; plants having normal (*Et*), colored seeds are one half A^1/A^2 and one half A^2/a; in cases in which it was not possible to distinguish the aleurone color effect of A^1 from that of A^2, the two latter types were not separable at planting and plants in the mixed progeny remained unidentified until harvest.

According to the evidence reported in the chapter dealing with allelic comparisons the *A* alleles are sharply divided on the basis of their effects on *P*-determined pericarp color. It will be remembered that the alleles which determine a distinctly intermediate plant phenotype produce a brown pericarp. In addition, A^{br}, A^{rb} and A^b always are associated with brown pericarp color while all other alleles of the purple class as listed in table 1 produce a red pericarp color. It is not possible to distinguish between alleles of the brown-pericarp class on the basis of the pericarp phenotype itself. The evidence from studies involving combinations of alleles, however, indicates that they are separable into two classes based on a dominance or lack of dominance of the brown pericarp effect over the red effect of *A*-st.

The dominance of pericarp color effect was studied in the progeny from the cross:

$$et\ A\text{-st}/et\ a \times Et\ A^{br}/et\ a.$$

The pericarp pigment of A^{br} plants, as usual, was brown, while that of *A*-st/*a*

individuals was red. A-st/A^{br} plants were found always to produce a red pericarp indistinguishable from that of the A-st/a plants. This is in agreement with the evidence of RHOADES (1941) who found the pericarp-color effect of A dominant to that of both A^{br} and A^{rb}.

Like A^{br}, A^{b} produces a brown pericarp color. Repeated attempts have been unsuccessful in distinguishing between these alleles either on the basis of their pericarp phenotype or their effects on plant color. That they nevertheless are distinct alleles is shown by their contrasted behavior in combinations with A-st. A-st/A^{b} plants always have brown pericarps in progenies in which A-st/a sib plants develop the usual red phenotype. From other progenies involving A^{b} it is evident that the dominance of brown-pericarp effect of this allele is not limited to its compound with A-st; combinations of A^{b} with A-D1 and with A-D2, both of which produce a red pericarp color in hybrids with a, also develop brown pericarp color. This difference in dominance behavior provides evidence of the individuality of A^{b} and A^{br} which is not evident from the comparison of these alleles in recessive a backgrounds.

The inconsistencies in the pericarp-color dominance relations in combinations of A-st with the alleles of the purple class are reflected in its combinations with alleles of the intermediate class. In combinations of A-st with A^{w}-D1 and A^{w}-U1, both of which determine brown pericarp color in hemizygous plants, the red color effect of A-st is dominant. The action of the A^{d} alleles is in direct contrast to that of A^{w}-D1 and A^{w}-U1. The former alleles, in spite of their association with decidedly intermediate plant and aleurone pigmentation, show complete dominance over A-st in pericarp color effect; plants representing combinations of A-st with a^{p}, A^{d}-41 or A^{d}-31 always develop brown pericarp color in progenies in which A-st/a sibs produce the usual red phenotype Nor is the dominant effect limited to combinations with A-st; in progenies involving a^{p} in combination with all of the "red pericarp" alleles of table 1, the pericarp effect of a^{p} is completely dominant.

Plant-Color Effects

The dominance of the pericarp-color effect of a^{p} in combinations with the alleles which determined red pericarp color is complete. So far as the eye can judge the brown phenotype of such compounds is indistinguishable from that of a^{p}/a individuals. The plant color phenotype of these combinations does not indicate a similar degree of dominance of a^{p}. It is possible, however, in all such combinations, to show a general effect of a^{p} resulting in some degree of intermediacy in plant color; since such compounds show reduced anthocyanin pigmentation in comparison with the heterozygotes of the "purple" alleles with a, the phenomenon has been termed "competition."

The competitive effect of a^{p} in combination with A^{br}, first reported by STADLER (1943), has been repeatedly confirmed in progenies from crosses of the following type:

$$et\ A^{br}/et\ a \times Et\ a^{p}/et\ a.$$

From this cross individuals having the genetic constitutions A^{br}/a and A^{br}/a^{p} were planted in separated progenies for comparison purposes. A^{br}/a plants are distinguishable from A^{br}/a^{p} plants in almost every vegetative region subject to coloration by the red and brown pigments; whereas in plants of the former type the sheaths, husks, rachis, glumes and anthers are purple in appearance, A^{br}/a^{p} plants show much weakened development of anthocyanin and a decidedly brownish-red cast in these regions. These differences, which are evident in the sheaths of plants in the five- or six-leaf stage, are present at flowering in all the pigmented regions and persist throughout later growth stages. The brownish appearance of A^{br}/a^{p} plants is most striking in the tassel glumes where there is a close approach to the phenotype of a^{p}/a plants. Over a period of three years, twelve separate progenies which permitted a comparison between A^{br}/a and A^{br}/a^{p} plants have been observed. In every one of these a competitive effect of the a^{p} allele was apparent. In those progenies which offered the opportunity for objective classification of unknown individuals, the genotypes were separated without error on the basis of the competitive effect.

The gene a^{p} has been tested for competitive behavior in combinations with A^{rb}. Observations of seven progenies involving the comparison of A^{rb}/a^{p} with A^{rb}/a plants indicate the competitive effect of a^{p} to be as pronounced in its combination with A^{rb} as it is with A^{br}, and fully reliable separations of plants of unknown genotypes are possible.

While the competitive effect of a^{p} in combinations with A^{br} and A^{rb} produces distinctly intermediate plant color phenotype, in combinations with other "purple" alleles, a^{p} has been found not to have so drastic an effect. For example, comparisons at maturity between A-st/a and A-st/a^{p} plants showed no observable differences in any of the pigmented regions of the plant. However, the chemical spot test, applied to husk tissue extracts of these plants clearly indicates a difference; spot tests of A-st/a^{p} extracts are distinctly more yellow-green than those of A-st/a extracts and these are readily classifiable as unknowns.

More recently, several other "purple" alleles have been shown to respond to the competitive effect of a^{p} to a degree permitting the visual separation of the combinations with a^{p} from the corresponding heterozygotes with a. This was accomplished through the use of objective classification. The method, as applied in this instance, may be illustrated by considering the experiments involving A-st. From the cross:

$$et\ A\text{-st}/et\ a \times Et\ a^{p}/et\ a,$$

A-st/a and A-st/a^{p} plants were grown in separated progenies for purposes of comparison. In addition a mixture of plants of both types was grown. Plants in the mixed progeny were classified as heterozygotes with either a^{p} or with a on the basis of pigmentation differences previously observed between the plants of known genotype. The correct constitutions of the unknown plants were determined by endosperm and pericarp phenotypes at maturity. A sta-

tistical basis for the verification or contradiction of the competitive effect of a^p was thus provided. In addition to *A*-st, the alleles, A^{br}, *A*-D6, *A*-D7, *A*-K1, *A*-K2, and *A*-K3 were tested for response to a^p by the objective classification method. The latter three alleles, not referred to previously, were extracted from Indian stocks which were obtained from southwestern states; they produce purple plant and aleurone color in combinations with recessive *a*. Of these comparisons, only the a^p combination with A^{br} produced plants showing a drastic and general weakened effect with respect to the development of anthocyanin, thus confirming the observations reported above. Casual observation of the plants representing combinations of the six remaining alleles with a^p showed no differences when compared with their respective heterozygotes with *a*. On closer examination it was noted that plants heterozygous for a^p showed slightly maroon-colored tassel glumes at flowering in contrast to the deeper purple appearance of these organs in the plants heterozygous for *a*. Objective classification of unknown progenies was attempted on the basis of these differences and in every case, verification of genetic constitutions at harvesting showed a high degree of accuracy in placements. The results of these classifications are presented in table 3. They indicate a general competitive action for a^p.

TABLE 3

The results of objective classifications of progenies planted from purple seeds from crosses of the type:
et A^1/*et a* × *Et* a^p/*et a*

A^1 ALLELE	PROGENY TOTAL	TOTAL CLASSED CORRECTLY	CLASSED CORRECTLY		CLASSED INCORRECTLY		P
			A^1/a	A^1/a^p	A^1/a	A^1/a^p	
A-st	15	14	7	7	1	0	<0.01
A^{br}	15	15	8	7	0	0	<0.01
A-D6	10	9	3	6	1	0	<0.02
A-D7	7	7	5	2	0	0	<0.01
A-K1	10	9	4	5	0	1	<0.02
A-K2	10	10	5	5	0	0	<0.01
A-K3	8	8	3	5	0	0	<0.01

As noted previously, in its lack of dosage effect and its dominance of brown pericarp effect a^p resembles the alleles A^d-41 and A^d-31. Experiments were conducted to determine whether these latter alleles have also the competitive effect in common with a^p. Because of its more pronounced response to competitive action by a^p, the allele A^{br} was chosen for the combinations. Several progenies providing a comparison of A^{br}/a and A^{br}/A^d-41 were grown from crosses of the following type:

$$et\ A^{br}/et\ a \times Et\ A^d\text{-}41/et\ a.$$

Similar crosses involving A^d-31 furnished progenies in which A^{br}/a and A^{br}/A^d-31 plants could be compared. Both of these alleles exhibit a competi-

tive effect in these backgrounds; plants having these alleles in combination with A^{br} are weakened in anthocyanin development in sheaths, husks, glumes, and anthers in comparison with A^{br}/a plants. Furthermore, to the extent that repeated observations in non-sib progenies may be relied upon, A^{d}-41 and A^{d}-31 differ from a^{p} and from each other in the degree of intermediacy of plant color produced in combinations with A^{br}. The direction of these differences appears to be a close parallel to that of the differences which exist between the three competing alleles when compared with each other as heterozygotes with *a*. a^{p}, which is the lowest of these three alleles on the scale of anthocyanin production, produces the most drastic reduction of anthocyanin in combination with A^{br}; A^{d}-31, the most potent in the production of anthocyanin, produces in combination with A^{br} the least reduction in anthocyanin; A^{d}-41 is, with respect to a^{p} and A^{d}-31, intermediate in plant color effect and this is true also of its competitive effect with A^{br}.

The competitive action of the alleles of South American origin is not shared by those of North American extraction. The allele A^{br} was employed in experiments to test for competitive effects of A^{w}-D1 and A^{w}-U1. Both alleles are negative in this regard; plants having the genetic constitution A^{br}/A^{w}-D1 are indistinguishable from A^{br}/a individuals and the same is true of the A^{br}/A^{w}-U1 plants. The lack of such an effect for these alleles is the third major source of evidence distinguishing A^{w}-D1 and A^{w}-U1 from a^{p}, A^{d}-41 and A^{d}-31; it will be remembered that these alleles were divided in the same manner into separate categories of action on the basis of the evidence from studies on dosage and the dominance of pericarp color effect.

Microscopic Evidence Concerning the Competitive Effect of a^{p}

From visual examination of the A^{br}/a^{p} plant it is evident that the quantity of purple pigment is much reduced over that in the A^{br}/a plant. It is not equally certain from macroscopic comparison that there is a corresponding increase in the brown pigment of the A^{br}/a^{p} plant, since it is reasonable to suppose that the reduction of purple pigment in the latter type merely displays to better advantage the brown pigment which may be considered to occur to the same extent in both types. Whether or not there is a change in level of brown pigment associated with the competitive effect is a point of considerable significance in connection with any interpretation of "competition" in terms of genic action at the biochemical level.

Microscopic studies have established that the A^{br}/a^{p} plant not only has less anthocyanin than its sib A^{br}/a plant but also has greater capacity for the production of brown pigment. In a progeny involving A^{br}/a^{p} and A^{br}/a plants, microscopic study of the distribution of pigments in the sheath tissues at the flowering stage revealed the absence of brown pigment in all plants of the latter type. At the same stage of development, sheaths of A^{br}/a^{p} plants showed a heavy deposition of brown pigment in cell walls. Moreover, as would be predicted from their macroscopic appearance, the pigment-bearing cells of A^{br}/a^{p} plants never develop more than a faint rose color in the vacuoles whereas corresponding tissues of the A^{br}/a plants are deep purple at maturity.

Much the same evidence, though differing in degree of effect, was obtained from a study of A-st/a^p and A-st/a plants. In this case, sheaths of the former type were devoid of brown pigment at the flowering stage but considerable brown pigment was present in sheath tissues ten days after pollination. Sheath tissues of A-st/a plants failed altogether in the production of brown pigment.

DISCUSSION

The results of the genetic studies are summarized in table 4. If the phenotypes produced in the several sites for A action are considered without regard to the evidence from dosage and dominance studies it appears that there are

TABLE 4

Summary of the results of the genetic studies

ALLELE	PHENOTYPE OF HETEROZYGOTE WITH a			GEOGRAPHIC ORIGIN	CUMULATIVE EFFECT WITH INCREASING DOSES	"COMPETITION" (IN COMPOUNDS WITH A^{br})
	PLANT	ALEURONE	PERICARP*			
A-st	purple	purple	red	North America	0	—
A-D†	"	"	"	"	0	—
A^b	dilute purple	"	dominant brown	South America	0	—
A^{rb}	"	"	recessive red-brown	North America	0	—
A^{br}	"	"	recessive brown	"	+	−
A^w-D1	pale++	pale++	"	"	+	0
A^w-U1	"	"	"	"	+	0
A^d-31	"	"	dominant brown	South America	0	+
A^d-41	pale+	pale+	"	"	0	+
a^p	pale	pale	"	"	0	+
a	brown	colorless	recessive brown	North America	0	0

* The dominance relationships of pericarp color effects are cited for combinations with A-st.
† The alleles A-D1, A-D2, A-D3, A-D6, and A-D7 are included under this general designation.

no exceptions to the linear action of the A alleles. Thus, if the alleles are arranged according to their effects on pigmentation of vegetative organs this order is found to represent also, without exceptions, their effects on the pigmentation of both the aleurone and pericarp tissues. It seems reasonable to inquire into the likelihood that these differences do not reside at the A locus but might instead be attributable to modifier genes closely linked with A. Such an explanation could not account for the difference in effect between a parent allele and a mutant obtained from it since the neighboring genes in the A linkage group should be identical for the two chromosomes involved, unless it is assumed that the parent A allele and modifier have, in the several cases of pertinence here, mutated simultaneously. In those comparisons involving A alleles which occur in chromosome complements having varied geographical

backgrounds it would be expected that many modifiers affecting pigmentation should segregate. Since, however, in the present studies two *A* alleles undergoing comparison have in all cases been brought into the same cell and have been compared subsequently in backcross progenies only, it would be expected that modifier genes, if not linked with *A*, should segregate equally among A^1/a and A^2/a plants. Slight differences among plants having identical *A* genotypes may be observed in almost all backcross progenies. Indeed the effects of modifier genes have made the task of identifying differences between the *A* alleles a much more difficult one and where such differences are relatively slight may prevent their identification altogether. It follows that the various levels of effect attributed to the *A* alleles (table 4) have been established, not in genetic backgrounds which evidenced no effects of modifiers, but in progenies in which the relatively greater differences in effect linked with the *A* alleles were superimposed on a background of varying effects due to segregating modifiers. If it is considered that these differences of greatest magnitude are to be attributed to segregating alleles, other than those at the *A* locus, it must be assumed that only those modifier genes of extreme effect are linked with *A*. This hypothesis, as an alternative to the one which would assign such differences to alleles of *A*, is the more complicated one, and the one least susceptible of analysis at the present time.

The evidence from the experiments on gene dosage and compounds adds to the list of distinguishable *A* alleles. The gene A^b is unique among alleles of the purple group in its dominance of brown pericarp effect. By reason of the same effect and the failure to show a cumulative effect with dosage A^d-31 is distinguishable from A^w-D1 and A^w-U1. The experiments reveal several other cases of non-linear action. The alleles associated with intermediate expression, all of which are characterized by purple and brown plant pigments and brown pericarp color, are separable into two distinct groups. In one of these are A^w-D1 and A^w-U1, characterized by cumulative effect with increased dosage, by the completely recessive nature (in compounds with *A*-st) of their brown-pericarp effect, and by the absence of a "competition" effect in compounds with A^{br} and the alleles of the purple class. In the other group are a^p, A^d-41 and A^d-31, which fail to show any changed effects with increasing doses, are completely dominant to *A*-st in their brown-pericarp effect, and show a "competition" effect in compounds. In the terminology of MULLER (1932) the alleles of the second group are antimorphic. That is to say these alleles when homozygous or in heterozygous combination with an amorph (recessive *a* in this case) produce some degree of type effect, yet produce an inhibition of type effect in combinations with other hypomorphs (A^{br}, A^{rb}, A^w-D1 and A^w-U1) or even with the wild type allele itself, *A*-st. This designation applies also to A^b which produces a weaker plant-color phenotype than *A*-st but is completely dominant to the latter in its brown-pericarp effect.

With the exception of A^b, a^p, A^d-41 and A^d-31, the action of all of the alleles listed in table 4 is consistent with the hypothesis to which reference has been made in a preceding chapter. According to this scheme (figure 1), and considering the pigmentation produced in vegetative regions of the plant, the *A*

alleles are concerned with the production of an agent which is capable of transforming a cellular substrate, S, into an immediate product, P, which is a precursor of the purple pigment. Different *A* alleles may be considered to determine correspondingly varying rates of this reaction. Since the production of

FIGURE 1.—A simple scheme of *A* action. S, cellular substrate; A, genic agent; P, product.

brown pigment is not associated in a positive way with action of the *A* alleles, and, furthermore, is negatively correlated with the production of purple pigment, it is reasonable to consider that substrate (S) is a precursor of both the brown and purple pigments and further that its supply is limiting. It does not follow that only these two reaction systems are in competition for the supply of S; in the absence of any knowledge concerning the chemical nature of S its rôle as a precursor of still other end-products of phytosynthesis may not be discounted. In the case of *A*-st it is reasonable to suppose that the transformation of S to P is "strong" enough to result in the complete absence of brown pigment. Conversely, in the absence of an *A* allele, transformation of S to P may be reasoned not to occur and hence to result in the complete absence of purple pigment. In the aleurone cells where no pigment is produced alternatively to anthocyanin, it is reasonable to suppose that the alternative end-product is colorless. Regarding pericarp phenotype, it may be reasoned along analogous lines that the *A* alleles produce from a substance S a precursor necessary in amounts above a certain threshold for the development of red pigment (not an anthocyanin.) The production, through the effect of an *A* allele, of this precursor at a rate such that the threshold value is not attained results in the manifestation of a brown-pericarp phenotype. In the case of the allele A^{rb}, which produces a red-brown pericarp color, the threshold required for the production of red pericarp pigment appears to be barely exceeded.

For purposes of illustration some of the results obtained with A^{w}-D1 may be considered. Two doses of this allele produce more anthocyanin and less brown pigment than one dose. Expressed in terms of the simple scheme described above, two doses of the A^{w}-D1 gene may be considered to accomplish a greater conversion of S to anthocyanin precursor than one dose and the increased level of precursor is followed by increased anthocyanin production. The increased utilization of S by the two doses of A^{w}-D1 would leave less substrate available for the production of brown pigment which is visibly reduced in amount in the homozygote. In the compound with A^{br}, A^{w}-D1 may be considered to accomplish the transformation of S into anthocyanin precursor at a rate greater than that accomplished by the A^{br} allele alone. Actual observa-

tions have not been sufficiently critical to measure an increase in anthocyanin in A^{br}/A^{w}-D1 compound as compared with the A^{br}/a plant, but certainly plants of the former type are no less pigmented with anthocyanin than those of the latter type. Likewise, in combinations with A-st, the A^{w}-D1 gene may be considered to utilize substrate, S, to the same end as, and in addition to, that utilized by A-st. Such compounds show complete dominance of A-st in plant, aleurone and pericarp color effects. In the same manner, the behavior of the other alleles listed in table 4 (excepting A^{b}, a^{p}, A^{d}-41 and A^{d}-31) may satisfactorily be accounted for by a simple correspondence between the gene and a single primary reaction. Figure 1 is intended to represent such a relationship only in barest outline.

The action of the intermediate alleles a^{p}, A^{d}-41 and A^{d}-31 cannot be fitted to this scheme. With regard to each of several phases of the action of these alleles, results have been obtained which are entirely unaccounted for by the simple gene product-substrate interaction discussed above: (1) although these alleles are intermediate in their effects, two doses of any one of them produce a phenotype indistinguishable from that produced by one dose. If the anthocyanin produced by these types is identical with that produced by the alleles of the first group, its concentration is certainly far below saturation and a lack of dosage effect would not be expected on the basis of the simple hypothesis. (2) Heterozygotes of these alleles with A^{br} show a reduction in amount of anthocyanin and an increase in the amount of brown pigment in comparison with A^{br}/a individuals. The allele a^{p} has the same "competition" effect in its combinations with the alleles of the purple class. This effect is not in agreement with expectations from the simple scheme which calls for increased type effect from the respective compounds. (3) The brown pericarp effect of a^{p}, A^{d}-41, A^{d}-31 and A^{b} is dominant to the red pericarp effect of A-st. Expectations would be exactly the opposite on the basis of the primary-reaction scheme described above.

These digressions from a simple gene product-substrate relationship must be accounted for by any hypothesis which lays claim to an accurate representation of the action of the A alleles.

Both WRIGHT (1941) and STERN (1943) have sought modification of the single, primary reaction hypothesis to explain the antimorphic effects of genes. The former has pointed out the possibility of explaining antimorphic action by (1) assuming that the alleles in a given series may react with a substrate which is in limited supply to produce qualitatively different products having different efficiencies with respect to the production of the type effect, or (2) by considering that the genic agent reacts with a substrate to produce an intermediate product which then reacts to produce the product, P; non-linear or antimorphic effects of some members of an allelic series may thus be explained by assuming that the alleles may differ independently in the rate constants for the two reactions in which the single genic agent is involved. These assumptions form the basis for a scheme proposed by STERN, who has suggested that with respect to its substrate interactions the gene or gene-product may have

two properties: (1) a combining power, (c), defined as the ability to react with substrate, and (2) an efficiency factor, (e), defined as the relative effectiveness of the allele in producing type effect from a unit amount of substrate. Two alleles may differ in their efficiency factors for one or both of two reasons: (1) the product formed from the substrate may be different for each allele, and these products may differ in their potency for the production of type effect, or (2) the product formed in the case of both alleles is the same, but the relative amounts of product which they form are different. There appear to be only slight differences between the assumptions made by STERN and those of WRIGHT; combining power may be thought of as equivalent to the rate constant for the first reaction; differences in efficiency as defined by STERN would include the possibilities of differences in rate constants for the second reaction and of qualitative differences in final product. It is intended to consider the evidence on the *A* alleles, particularly that of the alleles of the second group, in the light of these hypotheses and to show that the latter are inadequate to explain the results.

Since the alleles a^p, A^d-41 and A^d-31 behaved alike in the studies on dosage and compounds, only one of these, a^p, will be considered in weighing the merits of these hypotheses.

First hypothesis: Both A^{br} and a^p are concerned with the transformation of limited substrate, S, to an identical product, P, which is essential to the final development of anthocyanin. a^p, however, has greater combining capacity and a lower efficiency than A^{br}. A system of chain reactions leading to the production of brown pigment and not involving the action of *A* genic agent competes for the supply of S.

According to this scheme, since the a^p gene utilizes a large amount of S (otherwise available to the A^{br} gene for use in an efficient, anthocyanin-producing reaction) in the inefficient production of anthocyanin, it is expected that A^{br}/a^p cells would have less anthocyanin than A^{br}/a cells. This agrees with observation. However, since it is expected that A^{br} and a^p together would utilize more substrate than A^{br} alone, there should be less S available for the production of brown pigment in A^{br}/a^p than in A^{br}/a cells. Observations do not bear out this expectancy; A^{br}/a^p plants have more brown pigment than A^{br}/a sibs. Furthermore, regardless of how inefficient the a^p gene may be in its conversion of substrate, S, to P (anthocyanin precursor), two doses of a^p would be expected to utilize more S and result, therefore, in the production of greater amounts of P than one dose of the same gene (this follows from the hypothesis above, according to which S not transformed to P is available for the production of brown pigment.) This also is not borne out by observations; a^p/a^p plants are not distinguishable from a^p/a-X1 plants. This hypothesis must, therefore, on several counts, be considered inadequate to explain the results.

Second hypothesis: The product (P) produced by the reaction of substrate, S, with A^{br} genic agent is not identical with that produced by a^p action. P, the immediate product of a^p action is relatively inefficient with respect to the expression of type effect. Combining and efficiency values may but need not

differ in the cases of A^{br} and a^{p}. A system of chain reactions leading to the production of brown pigment and not involving the action of A genic agent competes for the supply of S.

As before, the expectancies based on this hypothesis do not account for the relatively greater production of brown pigment by the A^{br}/a^{p} plant as compared with the A^{br}/a plant, nor would the similarity between a^{p}/a^{p} and a^{p}/a-X1 plants be expected.

A modification of the second hypothesis might be considered. It may be reasoned that the A^{br} gene substance reacts with substrate to produce an anthocyanin precursor, whereas the a^{p} genic agent reacts with the same substrate to produce a precursor of brown pigment which may or may not be identical with the brown pigment associated with the a a genotype. This would account for the increased production of brown pigment by the A^{br}/a^{p} plant relative to that produced by A^{br}/a plants. On this basis, however, a^{p}/a-X1 or a^{p}/a^{p} plants would not be expected to show anthocyanin pigmentation. These types do, in fact, develop anthocyanin, and there is no less in a^{p}/a^{p} than in a^{p}/a-X1 individuals.

The contradictions to these hypotheses inherent in the observed results have led to the consideration of another scheme which appears to resolve the data. According to this hypothesis the a^{p} gene furthers the synthesis of both anthocyanin and brown pigment while the A^{br} gene produces anthocyanin precursor only. In terms of substrate conversion, the a^{p} genic substance may be considered to utilize substrate and transform it into two qualitatively different products, one of them a precursor of brown pigment, the other a precursor of anthocyanin. In the case of a^{p}, a disproportionately large amount of substrate, S, is transformed into the precursor of brown pigment. A^{br}, on the other hand, may be considered to transform S into the single product, anthocyanin precursor. Accordingly, A^{br}/a^{p} individuals have less anthocyanin than A^{br}/a plants because in the former type, the a^{p} gene, which is inefficient in its production of anthocyanin precursor, is in competition with the A^{br} gene for limited substrate. Furthermore, since the a^{p} gene transforms substrate into precursors of red and of brown pigment, A^{br}/a^{p} plants would be expected to produce more of the latter than A^{br}/a plants. This is in agreement with observation. The hypothesis also permits an explanation of the absence of a cumulative effect with increased dosage of the a^{p} gene, a fact which was unaccounted for by the hypotheses discussed above. If the a^{p} gene is considered to convert substrate into two products, one a precursor of anthocyanin, the other a precursor of brown pigment, and if one dose of a^{p} transforms all the substrate available, then two doses of the a^{p} gene would be expected to produce neither more nor less of the red and brown pigment precursors than one dose.

To account for the behavior of A^{d}-41, A^{d}-31 and A^{b} on the basis of this hypothesis requires the assumption that these alleles differ from a^{p} and from each other in the relative amounts of the red and brown pigment precursors which they produce from a unit quantity of substrate. Thus, A^{d}-31, which

produces anthocyanin much more abundantly than a^p, but like the latter allele shows no cumulative effect with increasing doses, may be thought of as producing from the substrate a greater amount of anthocyanin precursor relative to the amount of brown-pigment precursor than in the case of a^p. If, as was assumed in the case of a^p, the A^d-31 gene in one dose exhausts the available substrate, this would account for the lack of a dosage effect.

In the case of A^b, cumulative effect with increasing doses and behavior in compounds is being studied and it remains to be seen whether the action of this gene in such experiments resembles that of a^p. The dominance of its brown pericarp effect relative to the red effect of A-st is distinctly antimorphic and provides a further test of the hypothesis. It may be reasoned that A^b resembles the a^p alleles in its substrate-transformation relations and differs from them only with respect to the relative amounts of purple- and brown-pigment precursors which it determines. If it is assumed that the amount of purple pigment precursor produced is large with respect to the amount of brown-pigment precursor, the dilute-purple phenotype of A^b/a plants is explained. Furthermore, in the compound A-st/A^b, the A^b gene will be in competition with A-st for limited substrate, which it uses less efficiently than A-st in the production of red pigment precursor. The resulting lowered production of red pigment precursor in the A^{br}/A-st individual as compared to that of the A-st/a plant may cause it to fall below the threshold value required for the expression of red pericarp color; in this way, the brown pericarp color of the A^{br}/A-st plant may be accounted for. In the same way, the dominance of the brown-pericarp effect of all of the alleles of the second group may be explained.

The presence of several different levels of action among alleles of the second group offers a contradiction to an alternative hypothesis which might be considered to explain their antimorphic effects. According to this hypothesis these alleles may be considered to convert S into a single product, (different from P), which serves as a precursor of both purple and brown pigments. This would explain the lack of dosage effect (if S is exhausted by one dose) and the "competition" effect for any single one of these alleles, but it is not possible on this basis to explain the association of red and brown pigments in different ratios with the several alleles of this group unless additional assumptions regarding saturation effects in succeeding chain reactions are invoked.

The hypothesis which purports to satisfy the data now available places the A alleles in two distinct groups, with respect to gene action. In one group are those alleles of North American origin (table 4): A-st, A-D1, A-D2, A-D3, A-D6, A-D7, A^{br}, A^{rb}, A^w-U1, A^w-D1, and a. The action of these alleles may be represented as concerned with the transformation of a cellular substrate, S, to a precursor of purple pigment. Differences between these alleles may be explained by assuming that they determine different rates of a single reaction. In another group are those alleles which have a South American origin: A^b, a^p, A^d-41 and A^d-31. These behave as if they were concerned with the production of both brown- and purple-pigment precursors from the cellular substrate, S.

In the development of the foregoing hypothesis it is desirable, if not obliga-

tory, that some representation of the scheme be made in terms of interaction between genic agent and cellular substrate. It may be considered that a^p genic agent is specific in its affinity for a single substrate (figure 2a) in which case it is necessary to assume that the agent produces from a single substrate combination, the two immediate products, P and P′. According to an alternative view (figure 2b) the idea of a single genic agent is retained but it is pro-

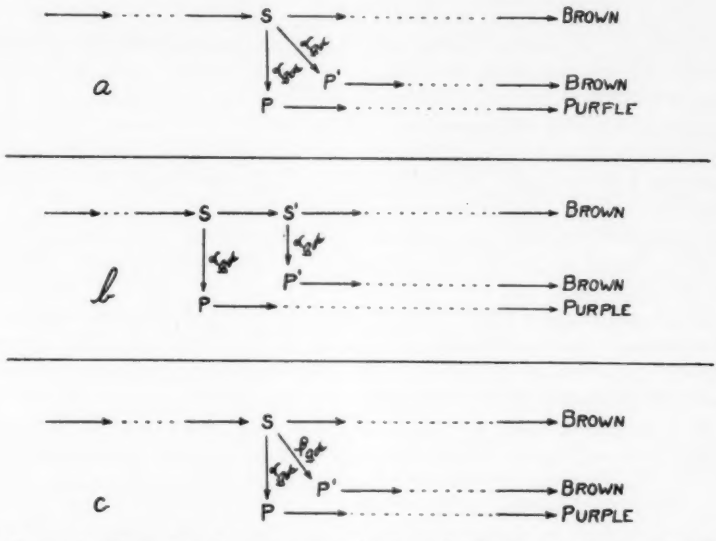

FIGURE 2.—Alternative schemes to account for the production, by a^p genic agent, of two immediate products. a, single agent ($\alpha_a{}^p$) and single substrate (S); b, single agent, two substrates (S and S′); c, two agents ($\alpha_a{}^p$ and $\beta_a{}^p$) and a single substrate.

posed that the agent enters into combination with two substrates, S and S′, which may differ by a single step. Two different products, P and P′, are produced by the interaction of the agent with the two closely related substrates. Thus the difference between P and P′ is not determined by divergent action of a single agent but is inherent in the difference between S and S′. On this basis the differences between the several alleles of the second group may be considered to reside in a qualitative difference between the agents produced such that one of the substrate reactions is enhanced or reduced relative to the other. If, as a third possibility, the a^p gene is held to consist of two, physically distinct units (alpha and beta components), it may be considered that P and P′ are produced from a single substrate by the action of two different agents which in turn derive from closely linked determinants (figure 2c).

It is not a purpose of this paper to weigh the relative merits of these schemes or of a number of others which might be proposed, nor would it be possible to do so objectively in terms of any data now at hand. It is intended rather to

emphasize that the data are not resolvable in terms of a single primary reaction for all alleles, and that, unless the hypothesis of closely linked genes is favored, it is necessary to view the action of a^p and its alleles of similar behavior as being in control of a two-dimensional reaction system. It is apparent, also, that such a scheme must involve independent reactions rather than separate phases of a single reaction.

SUMMARY

Histological studies of sheaths of maize plants carrying different *A* alleles show an inverse relation between the amounts of purple and brown pigments as well as a difference in their intracellular localization. *A*-st/*a* plants have deep purple cell vacuoles and are devoid of brown pigment, whereas *aa* plants have deep brown cell walls with no trace of vacuolar anthocyanin pigment. Plants carrying *A* alleles which determine intermediate phenotypes develop some brown wall pigment and show dilute purple vacuoles.

The brown pigment of *aa* plants is not the result of a positive action of the *a* allele; tissues which are homozygous deficient for the *A* locus show typical brown coloration.

The *A* alleles differ widely in their effects on anthocyanin pigmentation in aleurone and vegetative regions and in their effects on pericarp color. Seven distinct levels of action for alleles of this locus have been established in studies involving heterozygotes with recessive *a*.

With increasing doses (measured in a deficiency background) A^w-D1 and A^w-U1 have cumulative effects in both plant and aleurone tissues. In cells of the sheath, two doses of these alleles produce more purple and less brown pigment than one dose. The alleles a^p, A^d-41 and A^d-31 (of South American extraction) exhibit no dosage effect in either plant or endosperm tissue. The results of experiments using *a* instead of the deficiency are comparable in all respects to those from gene dosage studies.

Combinations of the alleles A^w-D1 and A^w-U1 (associated with intermediate plant and brown pericarp color) with alleles of the purple class show a dominance of the latter; these compounds develop deep purple vegetative parts and red pericarps. The alleles, a^p, A^d-41 and A^d-31 (of the intermediate class also) behave quite the opposite in combinations with alleles of the purple class; such compounds have less anthocyanin and more brown pigment than the purple types with which the combination was made and show complete dominance of the brown pericarp effect. A^b is also antimorphic in its effect on pericarp color; in compounds with *A*-st and other alleles associated with red pericarp color, the brown effect of A^b is completely dominant.

The divergent action of the alleles of South American origin (a^p, A^d-41, A^d-31 and A^b) is not explained by a simple hypothesis which considers the *A* alleles to accomplish different degrees of transformation of cellular substrate to a single product essential for the formation of purple pigment.

The data may be brought into agreement by assuming that the alleles of South American origin further the synthesis of both brown and purple pigment

while those of North American extraction act to convert the cellular substrate to a single product, a precursor of anthocyanin.

A one to one relation between gene and reaction may be defended by assuming that the alleles of dual action are compound determinants whose components are closely, perhaps inseparably, linked.

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